

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**In re application of:** Pastan et al.**Application No.** 10/495,663**Filed:** May 12, 2004**Confirmation No.** 1502**For:** GENE EXPRESSED IN PROSTATE CANCER  
AND METHODS OF USE**Examiner:** Minh-Tam Davis**Art Unit:** 1642**Attorney Reference No.** 4239-68238-01**SUBMITTED VIA EFS  
ON NOVEMBER 20, 2007**SUBMITTED VIA THE ELECTRONIC FILING SYSTEM (EFS)  
COMMISSIONER FOR PATENTSTRANSMITTAL LETTER

Enclosed for filing in the application referenced above are the following:

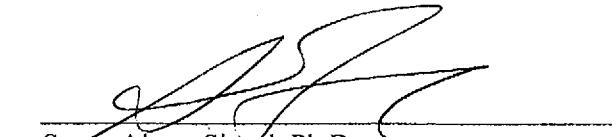
- Notice of Appeal From the Examiner to the Board of Patent Appeals and Interferences
- Pre-Appeal Brief Request for Review (5 pages)
- Exhibits A-H
- Authorization for electronic payment in the amount of \$510.00 from our Deposit Account No. 02-4550 is provided electronically.
- The Director is hereby authorized to charge any additional fees that may be required, or credit over-payment, to Deposit Account No. 02-4550.

Respectfully submitted,

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cc: Docketing; accounting

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COMMISSIONER FOR PATENTS**NOTICE OF APPEAL FROM THE EXAMINER  
TO THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Applicants hereby appeal to the Board from the decision of Examiner Minh-Tam Davis mailed August 22, 2007, finally rejecting claims 1, 17-21, 38, 46 and 48.

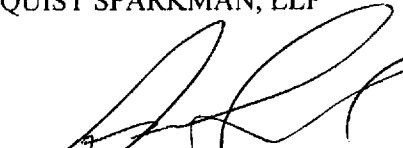
The fee of \$510.00 per 37 C.F.R. § 1.17(b) for filing this Notice of Appeal is being submitted herewith, via EFS. If an extension of time is required for filing this Notice of Appeal Please consider this a petition therefore. Please charge any additional fees that may be required in connection with filing this Notice of Appeal, any extension of time, or credit any overpayment, to Deposit Account No. 02-4550.

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**Attorney Reference No.** 4239-68238-01

**SUBMITTED VIA EFS  
ON NOVEMBER 20, 2007**

SUBMITTED VIA THE ELECTRONIC FILING SYSTEM (EFS)  
COMMISSIONER FOR PATENTS

**PRE-APPEAL BRIEF REQUEST FOR REVIEW**

Applicants request pre-appeal review of the final rejection in the referenced application. No amendments are being filed with this request. This request is submitted with a Notice of Appeal. The review is requested for the reasons stated below.

**Pending Claims**

Claims 1, 17-21, 38, 46 and 48 are under consideration. The pending claims (which are provided as Exhibit A) are directed to an isolated polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 1 and methods for detecting prostate cancer in a subject using antibodies that specifically bind a polypeptide that comprises the amino acid sequence set forth as SEQ ID NO: 1. Claims 6-16, 24-37, 39-41 and 43-45 are withdrawn from consideration as being drawn to a non-elected invention.

**Summary of the Outstanding Rejections**

Claims 1, 17-21, 38, 46 and 48 are rejected under 35 U.S.C. § 101 as allegedly the claimed polypeptides and methods are not supported by a specific, credible, substantial or well-established utility. Claims 1, 17-21, 38, 46 and 48 are rejected under 35 U.S.C. § 112, first paragraph as allegedly not being enabled by the specification. All of the rejections based on the prior art have been withdrawn; the pending claims are free of the prior art of record.

**Rejection Under 35 U.S.C. § 101**

Claims 1, 17-21, 38, 46 and 48 are rejected under 35 U.S.C. § 101 as allegedly the claimed polypeptides and methods are not supported by a specific, credible, substantial or well-established utility. The Office action acknowledges that it has been demonstrated that a polypeptide comprising SEQ ID NO: 1 is expressed both in normal prostate tissue and in prostate cancer. However, the Office action asserts

that because (1) a polypeptide comprising SEQ NO: 1 is not differentially expressed in primary prostate cancer (as compared to normal tissue) then it simply cannot be used to detect prostate cancer. In addition, antibodies cannot be used to detect metastatic prostate cancer because it is not predictable that cancer will express the polypeptide. The Office action further alleges that (2) detecting normal prostate cells is "not a specific and substantial utility, because it is not clear what practical use is for detecting normal prostate cells, and further because it is the property shared by numerous other prostate specific polypeptides" (see page 4). The Office action further alleges that the claimed protein (3) cannot be of use in treating prostate cancer, due to the unpredictability of cancer therapy. Each of these assertions are addressed individually below. Exhibit A is a listing of the pending claims. Exhibits B-D and G-H are of record in the present application (see the response submitted on June 11, 2007). Exhibit E and F are newly submitted.

*(1) Antibodies that bind SEQ ID NO: 1 specifically identify prostate cancer as prostate cancer has been shown to express this polypeptide:* Cancer is known to metastasize; prostate cancer metastasizes to bones, lymph nodes, rectum and bladder (see Wikipedia on Prostate Cancer, printout attached as Exhibit B, discussed in prior response). It is well known that the origin of a cancer can provide substantial insight into treatment methods.

Thus, expression of a polypeptide encoding SEQ ID NO: 1 can be used to identify a tumor in a tissue, such as the bone, lymph node, rectum or bladder as being of prostate or uterine origin. Indeed, the specification discloses that antibodies can be used to detect NGEP (SEQ ID NO: 1) expressing cells, to determine whether metastatic cells of a prostate cancer (or another PAGE4 expressing cancer) have metastasized to other areas of the body (see the specification at pages 52-53).

Antibodies are also routinely used in histological analysis, and are often sold as parts of a kit for the detection of cells in any biological sample (see the specification at pages 40-42). The antibodies disclosed herein could be used in routine histological analysis, such as to determine the presence of any prostate cells (from either a tumor or from a normal prostate) in a sample. Thus, there is a credible, specific, and substantial utility for the claimed polypeptides in the production of antibodies for the detection of prostate cancer metastasis.

Das et al. (Cancer Res. 67: 1594-1601, 2007, Exhibit C, of record) discloses that there are two forms of NGEP, a short form (SEQ ID NO: 1) and a long form, that includes SEQ ID NO: 1. Antibodies were produced that specifically bind the protein including SEQ ID NO: 1. These antibodies were used to detect this protein in extracts of normal prostate and prostate cancers (see Figures 1 and 2). Thus, Das et al. provide evidence documenting that polypeptides comprising SEQ ID NO: 1 continue to be expressed in prostate cancer.

In addition, Das et al., (poster presentation from the AACR meeting, 2007, Exhibit D, of record) describes the production of a panel of monoclonal and polyclonal antibodies that bind a polypeptide comprising SEQ ID NO: 1. The antibodies detected the presence of the protein (SEQ ID NO: 1) in

extracts of both normal prostate and prostate cancers (see Fig. 1E and the table adjacent to Fig. 1). Thus, antibodies can readily be produced to a polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 1, and these antibodies have been used to detect normal prostate and prostate cancer.

It has been clearly documented that polypeptides comprising SEQ ID NO: 1 are expressed in prostate cancer. Data has been presented documenting that antibodies that specifically bind SEQ ID NO: 1 can be obtained, and that these antibodies can be used to detect polypeptides comprising SEQ ID NO: 1 in prostate cancer cells. The allegation in the final Office action that a polypeptide comprising SEQ ID NO: 1 will not be expressed in metastatic prostate cancer, and thus that prostate cancer cells cannot be identified by the expression of this polypeptide, is unfounded and contradicted by the evidence of record.

*(2) The detection of prostate cells is a specific, credible and substantial utility:* Antibodies are also routinely used in histological analysis, and are often sold as parts of a kit for the detection of cells in any biological sample (see the specification at pages 40-42). As documented, proteins comprising SEQ ID NO: 1 can be used to produce antibodies that specifically bind SEQ ID NO: 1. The specification discloses that these antibodies can be used in routine histological analysis (see pages 40-42), such as to determine the presence of any prostate cells (from either a tumor or from a normal prostate) in a sample.

Antibodies to prostate cells are used to study the development and maturation of the prostate. This is exemplified in Exhibit E (Renneberg et al., J. Anat. 190: 343-349, 1997), which describes an immunohistochemical study of a prostate membrane specific protein during the development and maturation of the human prostate.

Antibodies that bind normal prostate are available as commercial products. Submitted herewith as Exhibit F are printouts of data sheets for antibodies that are currently being sold (AbCam, Prostate Secretory Protein/PSP antibody, protein produced in prostate, <http://abcom.com/index.html?datasheet+19070>; Abcam, PATE (prostate and testis expressed gene, [http://biocompare.com/matrixsc/3194/2/6/118846/PATE+...;](http://biocompare.com/matrixsc/3194/2/6/118846/PATE+...); Research Diagnostics, Inc, Prostate Specific Antigen (PSA), <http://researchd.com/miscabs/psa.htm>). The number of antibodies that bind antigens expressed in normal prostate cells that are for sale documents that a market demand exists for multiple antibodies that bind antigens expressed in normal prostate, and for methods to detect prostate cells by detecting prostate-specific protein expression.

Thus, the assertion that antigens that are expressed in normal cells cannot be of use is baseless. Applicants have documented that a protein with the amino acid sequence set forth as SEQ ID NO: 1 can be used to produce antibodies, and that these antibodies specifically bind prostate cells. The Applicants have also demonstrated that expression of protein comprising SEQ ID NO: 1 can be used to identify prostate cells. Methods to detect proteins in normal prostate cells are established in the art, and thus have real-world use and commercial value. Thus, a second specific, substantial and credible use has been demonstrated.

(3) *Polypeptides comprising SEQ ID NO: 1, and antibodies that bind these polypeptides, can be used for the treatment of cancer:* The Office action alleges that the claimed polypeptides cannot be of use to treat prostate cancer, because cancer immunotherapy is unpredictable. In the present application, a therapeutic use has been asserted, namely the use of the polypeptides for the treatment and or detection of cancer, such as prostate cancer (see the specification, pages 33-35).

Epitopes of other proteins can be used for the immunotherapy of cancer. For example, two mesothelial epitopes have been used to generate T cell lines that lyse pancreatic and ovarian tumor cells (Yokokawa et al., Clin. Canc. Res. 17: 6342-6351, 2005, Exhibit G). In addition, as disclosed in the specification (for example, see pages 37-40), the claimed polypeptides can be used to generate antibodies that can be used for the treatment of cancer. Targeted therapy for cancer that utilizes antibodies is well known in the art (see for example, Fan et al., Mol. Cancer Therap. 1: 595-600, 2002, Exhibit H). The Courts have found utility for therapeutic inventions despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition. The Federal Circuit, in *Cross v. Iizuka*, 753 F.2d 1040, 1051, 224 USPQ 739, 747-48 (Fed. Cir. 1985) found that data from *in vitro* testing that showed pharmacological activity could be used to support the utility of the claimed product. MPEP § 2107 states:

“As a general matter, evidence of pharmacological or other biological activity of a compound will be relevant to an asserted therapeutic use if there is a reasonable correlation between the activity in question and the asserted utility. *Cross v. Iizuka*, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985); *In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980); *Nelson v. Bowler*, 626 F.2d 853, 206 USPQ 881 (CCPA 1980). An applicant can establish this reasonable correlation by relying on statistically relevant data documenting the activity of a compound or composition, arguments or reasoning, documentary evidence (e.g., articles in scientific journals), or any combination thereof. The applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor does he or she have to provide actual evidence of success in treating humans where such a utility is asserted. Instead, as the courts have repeatedly held, all that is required is a reasonable correlation between the activity and the asserted use. *Nelson v. Bowler*, 626 F.2d 853, 857, 206 USPQ 881, 884 (CCPA 1980).” [emphasis added]

In the present case, there is a reasonable certainty that the claimed polypeptides can be used for the treatment of prostate cancer.

To satisfy the requirements of 35 U.S.C. § 101, an invention must be useful, and the specification must include specific information to make it apparent to one of skill in the art that the use is “specific,” “substantial” and “credible.” The present application discloses a polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 1, or a polypeptide including or consisting of 8 to 11 amino acids of SEQ ID NO: 1. The specification discloses that these polypeptides are of use to produce antibodies that can be used to detect normal prostate cells and prostate cancer, and can be used as a therapeutic agent.

Thus, at least three “real-world” uses have been documented that are specific, substantial, and credible. Any one of these utilities is sufficient to support patentability. Reconsideration and withdrawal of the rejection are requested.

**Rejection Under 35 U.S.C. § 112, First Paragraph**

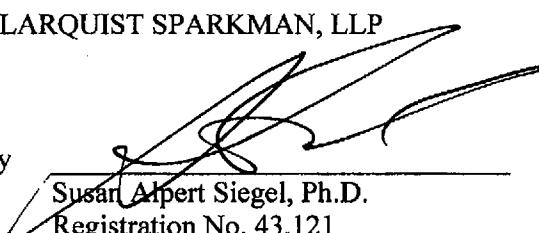
Claims 1, 17-21, 38, 46 and 48 are rejected under 35 U.S.C. § 112, first paragraph as allegedly not being enabled by the specification. The Office action states the “since the claimed invention is not supported by either a specific asserted or well established utility...one skilled in the art clearly would not know how to use the claimed invention.” The Office action simply states the same arguments and reasons set forth under 35 U.S.C. § 101 and applies them to the enablement rejection. Applicants respectfully disagree.

The Office action alleges that since there is no utility for the claimed polypeptides, it is impossible for one of skill in the art to make or use the claimed polypeptides. Applicants note that the production of polypeptides is routine for a skilled molecular biologist, and is described in the specification (see, for example, pages 22-26). In addition, there is a specific, credible and substantial utility for the claimed polypeptides, as discussed above. Methods of detecting prostate cancer are described in the specification (see pages 40-42). Moreover, evidence was presented (see Exhibits C and D) documenting that one of skill in the art, using the methods described in the specification, can use the claimed polypeptides (claims 1, 38, 46 and 48) to produce antibodies which can be used to detect cancer (claims 17-21) and normal prostate cells. It has been documented that one of skill in the art could make and use the claimed proteins to produce antibodies and use these antibodies to detect the expression of protein in both normal prostate and prostate cancer cells. Given the high level of one of skill in the art, the detailed guidance provided in the specification, and the documentation of reduction to practice, it is clear that the claimed methods are fully enabled. Reconsideration and withdrawal of the rejection are respectfully requested.

Respectfully submitted,

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**EXHIBIT A**

U.S. Patent Application No.: 10/495,663, filed May 12, 2004

Confirmation No.: 1502

Our Ref. No.: 4239-68238-01

Examiner: Minh-Tam Davis

First Named Inventor: Pastan

For: GENE EXPRESSED IN PROSTATE CANCER AND METHODS OF USE

Art Unit: 1642

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Listing of claims as pending:

1. (Previously Presented) An isolated polypeptide comprising an amino acid sequence set forth as SEQ ID NO:1.

2-5. (Canceled).

6. (Withdrawn) An isolated nucleic acid sequence encoding the polypeptide of claim 1.

7. (Withdrawn) The isolated nucleic acid sequence of claim 6, comprising a sequence as set forth as SEQ ID NO:2, or a degenerate variant thereof.

8. (Withdrawn) The isolated nucleic acid sequence of claim 6, operably linked to a promoter.

9. (Withdrawn) An expression vector comprising the nucleic acid sequence of claim 6.

10. (Withdrawn) A host cell transfected with the nucleic acid sequence of claim 6.

11. (Withdrawn) The host cell of claim 10, wherein the host cell is a mammalian cell.

12. (Withdrawn) An antibody that specifically binds the amino acid sequence set forth as SEQ ID NO: 1.

13. (Withdrawn) The antibody of claim 12, wherein the antibody is a monoclonal antibody.

14. (Withdrawn) The antibody of claim 12 comprising a detectable label.
15. (Withdrawn) The antibody of claim 12, wherein the label is a fluorescent, enzymatic or radioactive label.
16. (Withdrawn) The antibody of claim 12 conjugated to a toxin.
17. (Previously Presented) A method for detecting prostate cancer in a subject, comprising  
contacting a sample obtained from the subject with the antibody that specifically binds the polypeptide of claim 1 for a sufficient amount of time to form an immune complex;  
detecting the presence the immune complex, wherein the presence of an immune complex demonstrates the presence of prostate cancer in the subject.
18. (Original) The method of claim 17, wherein the sample is a biopsy, blood, serum, or urine sample.
19. (Original) The method of claim 17, wherein the sample is a biopsy sample of non-prostate origin.
20. (Original) The method of claim 17, wherein the antibody is labeled.
21. (Previously Presented) A method for detecting a prostate cancer in a subject, comprising  
contacting the sample with a labeled antibody that specifically binds the polypeptide of claim 1 for a sufficient amount of time to form an immune complex; and  
detecting the presence of the immune complex thereby detecting the presence of the prostate cancer.
- 22-23. (Canceled).

24. (Withdrawn) The method of claim 21, wherein detecting the expression of the polypeptide comprises detecting the presence of mRNA encoding polypeptide.

25. (Withdrawn) The method of claim 24, wherein detecting the presence of mRNA encoding the polypeptide comprises a Northern Blot analysis, an RNA dot blot, or a reverse transcriptase polymerase chain reaction (RT-PCR) assay.

26. (Withdrawn) A method for producing an immune response against a cell in a subject, comprising

administering to the subject a therapeutically effective amount of the polypeptide of claim 1, or a polynucleotide encoding the polypeptide,

wherein the cell expresses the polypeptide of claim 1, thereby producing the immune response against the cell.

27. (Withdrawn) The method of claim 26, wherein the immune response is a T cell response.

28. (Withdrawn) The method of claim 26, wherein the immune response is a B cell response.

29. (Withdrawn) The method of claim 26, wherein the subject has prostate cancer.

30. (Withdrawn) The method of claim 29, wherein the immune response decreases the growth of the prostate cancer.

31. (Withdrawn) A method for inhibiting the growth of a malignant cell expressing a polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 1, comprising,

(i) culturing cytotoxic T lymphocytes (CTLs) or CTL precursor cells with the polypeptide of claim 1 to produce activated CTLs or CTL precursors that recognize a cell expressing a polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 1, and

(ii) contacting the malignant cell with the activated CTLs or CTLs matured from the CTL precursors,  
thereby inhibiting the growth of the malignant cell.

32. (Withdrawn) A method for inhibiting the growth of a malignant cell comprising:  
contacting the malignant cell with an effective amount of a cell-growth inhibiting molecule, wherein the cell growth inhibiting molecule comprises an antibody which specifically binds the polypeptide of claim 1 covalently linked to an effector molecule which inhibits the growth of cells, thereby inhibiting the growth of the malignant cell,  
wherein the malignant cell expresses the polypeptide of claim 1.

33. (Withdrawn) The method of claim 32, wherein said antibody is a monoclonal antibody.

34. (Withdrawn) The method of claim 32, wherein the effector molecule is a chemotherapeutic agent.

35. (Withdrawn) The method of claim 32, wherein the effector molecule comprises a toxic moiety.

36. (Withdrawn) The method of claim 35, wherein the toxic moiety is selected from the group consisting of ricin A, abrin, diphtheria toxin or a subunit thereof, *Pseudomonas* exotoxin or a portion thereof, and botulinum toxins A through F.

37. (Withdrawn) The method of claim 35, wherein the *Pseudomonas* exotoxin is selected from the group consisting of PE35, PE37, PE38, and PE40.

38. (Previously Presented) A pharmaceutical composition comprising a therapeutically effective amount of the polypeptide of claim 1 in a pharmaceutically acceptable carrier.

39. (Withdrawn) A pharmaceutical composition comprising a therapeutically effective amount of the polynucleotide of claim 6 in a pharmaceutically acceptable carrier.

40. (Withdrawn) A pharmaceutical composition comprising a therapeutically effective amount of the antibody of claim 12 in a pharmaceutically acceptable carrier.

41. (Withdrawn) A method for reducing the number of prostate cancer cells in a subject, comprising

administering to the subject a therapeutically effective amount of the polypeptide of claim 1, wherein the administration of the polypeptide results in an immune response to the prostate cancer cells,

thereby reducing the number of prostate cancer cells in the subject.

42. (Canceled).

43. (Withdrawn) A method for reducing the number of prostate cancer cells in a subject, comprising

administering to the subject a therapeutically effective amount of the antibody of claim 16,

thereby reducing the number of prostate cancer cells in the subject.

44. (Withdrawn) A kit for detecting an polynucleotide encoding the polypeptide of claim 1 in a sample, comprising

an isolated nucleic acid sequence of at least ten nucleotides in length that specifically binds to SEQ ID NO:2 under highly stringent hybridization conditions; and

instructions for the use of the isolated nucleic acid sequence.

45. (Withdrawn) A kit for detecting a polypeptide in a sample, comprising  
a monoclonal antibody that specifically binds the amino acid sequence set forth as SEQ ID NO:1; and

instructions for the use of the antibody.

46. (Previously Presented) A polypeptide consisting of the amino acid sequence set forth as SEQ ID NO:1

47. (Canceled).

48. (Previously Presented) A method for detecting a prostate cancer or a prostate cell in a subject, comprising

contacting the sample with an antibody that specifically binds the polypeptide of claim 46 for a sufficient amount of time to form an immune complex; and

detecting the presence of the immune complex, thereby detecting the prostate cell or the prostate cancer in the subject.

49. (Previously Presented) The method of claim 17, wherein the sample is a biopsy, blood, serum, or urine sample.

50. (Previously Presented) The method of claim 17, wherein the sample is a biopsy sample of non-prostate origin.

51. (Previously Presented) The method of claim 17, wherein the antibody is labeled.

# Prostate cancer

From Wikipedia, the free encyclopedia

**Prostate cancer** is a disease in which cancer develops in the prostate, a gland in the male reproductive system. It occurs when cells of the prostate mutate and begin to multiply out of control. These cells may spread (metastasize) from the prostate to other parts of the body, especially the bones and lymph nodes. Prostate cancer may cause pain, difficulty in urinating, erectile dysfunction and other symptoms.

Rates of prostate cancer vary widely across the world. Although the rates vary widely between countries, it is least common in South and East Asia, more common in Europe, and most common in the United States.<sup>[1]</sup> According to the American Cancer Society, prostate cancer is least common among Asian men and most common among black men, with figures for white men in-between.<sup>[2][3]</sup> However, these high rates may be affected by increasing rates of detection.<sup>[4]</sup>

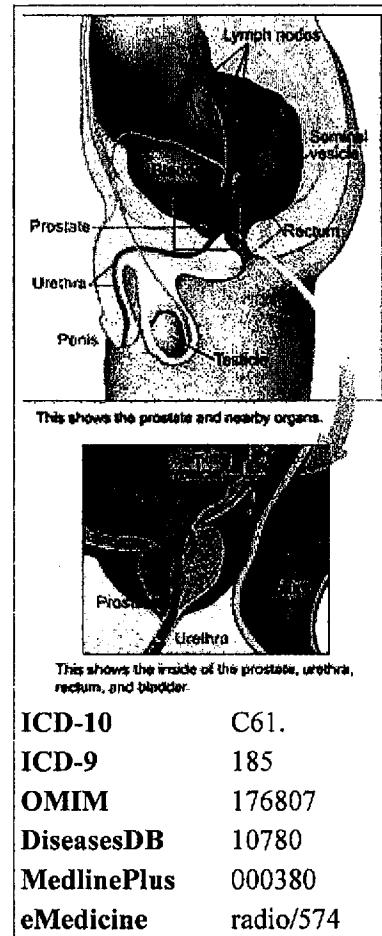
Prostate cancer develops most frequently in men over fifty. This cancer can occur only in men, as the prostate is exclusively of the male reproductive tract. It is the most common type of cancer in men in the United States, where it is responsible for more male deaths than any other cancer, except lung cancer. However, many men who develop prostate cancer never have symptoms, undergo no therapy, and eventually die of other causes. Many factors, including genetics and diet, have been implicated in the development of prostate cancer.

Prostate cancer is most often discovered by physical examination or by screening blood tests, such as the PSA (prostate specific antigen) test. There is some current concern about the accuracy of the PSA test and its usefulness. Suspected prostate cancer is typically confirmed by removing a piece of the prostate (biopsy) and examining it under a microscope. Further tests, such as X-rays and bone scans, may be performed to determine whether prostate cancer has spread.

Prostate cancer can be treated with surgery, radiation therapy, hormonal therapy, occasionally chemotherapy, proton therapy, or some combination of these. The age and underlying health of the man as well as the extent of spread, appearance under the microscope, and response of the cancer to initial treatment are important in determining the outcome of the disease. Since prostate cancer is a disease of older men, many will die of other causes before a slowly advancing prostate cancer can spread or cause symptoms. This makes treatment selection difficult.<sup>[5]</sup> The decision whether or not to treat localized prostate cancer (a tumor that is contained within the prostate) with curative intent is a patient trade-off between the expected beneficial and harmful effects in terms of patient survival and quality of life.

## Prostate cancer

Classification & external resources



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## Prostate

The prostate is a male reproductive organ which helps make and store seminal fluid. In adult men a typical prostate is about three centimeters long and weighs about twenty grams.<sup>[6]</sup> It is located in the pelvis, under the urinary bladder and in front of the rectum. The prostate surrounds part of the urethra, the tube that carries urine from the bladder during urination and semen during ejaculation.<sup>[7]</sup> Because of its location, prostate diseases often affect urination, ejaculation, and rarely defecation. The prostate contains many small glands which make about twenty percent of the fluid constituting semen.<sup>[8]</sup> In prostate cancer the cells of these prostate glands mutate into cancer cells. The prostate glands require male hormones, known as androgens, to work properly. Androgens include testosterone, which is made in the testes; dehydroepiandrosterone, made in the adrenal glands; and dihydrotestosterone, which is converted from testosterone within the prostate itself. Androgens are also responsible for secondary sex characteristics such as facial hair and increased muscle mass.

## Symptoms

Early prostate cancer usually causes no symptoms. Often it is diagnosed during the workup for an elevated PSA noticed during a routine checkup. Sometimes, however, prostate cancer does cause symptoms, often similar to those of diseases such as benign prostatic hypertrophy. These include frequent urination, increased urination at night, difficulty starting and maintaining a steady stream of urine, blood in the urine, and painful urination. Prostate cancer may also cause problems with sexual function, such as difficulty achieving erection

or painful ejaculation.<sup>[9]</sup>

Advanced prostate cancer may cause additional symptoms as the disease spreads to other parts of the body. The most common symptom is bone pain, often in the vertebrae (bones of the spine), pelvis or ribs, from cancer which has spread to these bones. Prostate cancer in the spine can also compress the spinal cord, causing leg weakness and urinary and fecal incontinence.<sup>[10]</sup>

## Pathophysiology

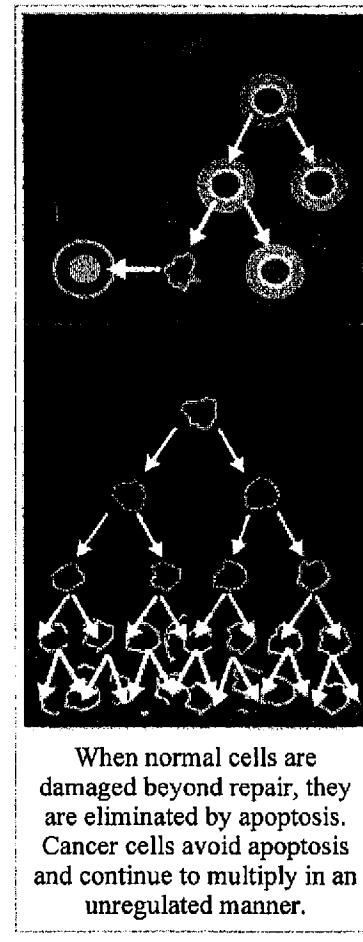
Prostate cancer is classified as an adenocarcinoma, or glandular cancer, that begins when normal semen-secreting prostate gland cells mutate into cancer cells. The region of prostate gland where the adenocarcinoma is most common is the peripheral zone. Initially, small clumps of cancer cells remain confined to otherwise normal prostate glands, a condition known as carcinoma in situ or prostatic intraepithelial neoplasia (PIN). Although there is no proof that PIN is a cancer precursor, it is closely associated with cancer. Over time these cancer cells begin to multiply and spread to the surrounding prostate tissue (the stroma) forming a tumor. Eventually, the tumor may grow large enough to invade nearby organs such as the seminal vesicles or the rectum, or the tumor cells may develop the ability to travel in the bloodstream and lymphatic system. Prostate cancer is considered a malignant tumor because it is a mass of cells which can invade other parts of the body. This invasion of other organs is called metastasis. Prostate cancer most commonly metastasizes to the bones, lymph nodes, rectum, and bladder.

## Etiology

The specific causes of prostate cancer remain unknown.<sup>[11]</sup> A man's risk of developing prostate cancer is related to his age, genetics, race, diet, lifestyle, medications, and other factors. The primary risk factor is age. Prostate cancer is uncommon in men less than 45, but becomes more common with advancing age. The average age at the time of diagnosis is 70.<sup>[12]</sup> However, many men never know they have prostate cancer. Autopsy studies of Chinese, German, Israeli, Jamaican, Swedish, and Ugandan men who died of other causes have found prostate cancer in thirty percent of men in their 50s, and in eighty percent of men in their 70s.<sup>[13]</sup> In the year 2005 in the United States, there were an estimated 230,000 new cases of prostate cancer and 30,000 deaths due to prostate cancer.<sup>[14]</sup>

A man's genetic background contributes to his risk of developing prostate cancer. This is suggested by an increased incidence of prostate cancer found in certain racial groups, in identical twins of men with prostate cancer, and in men with certain genes. In the United States, prostate cancer more commonly affects black men than white or Hispanic men, and is also more deadly in black men.<sup>[15]</sup> Men who have a brother or father with prostate cancer have twice the usual risk of developing prostate cancer.<sup>[16]</sup> Studies of twins in Scandinavia suggest that forty percent of prostate cancer risk can be explained by inherited factors.<sup>[17]</sup> However, no single gene is responsible for prostate cancer; many different genes have been implicated. Two genes (*BRCA1* and *BRCA2*) that are important risk factors for ovarian cancer and breast cancer in women have also been implicated in prostate cancer.<sup>[18]</sup>

Dietary amounts of certain foods, vitamins, and minerals can contribute to prostate cancer risk. Men with



When normal cells are damaged beyond repair, they are eliminated by apoptosis. Cancer cells avoid apoptosis and continue to multiply in an unregulated manner.

higher serum levels of the short-chain ω-6 fatty acid linoleic acid have higher rates of prostate cancer. However, the same series of studies showed that men with elevated levels of long-chain ω-3 (EPA and DHA) had lowered incidence.<sup>[19]</sup> A long-term study reports that "blood levels of trans fatty acids, in particular trans fats resulting from the hydrogenation of vegetable oils, are associated with an increased prostate cancer risk."<sup>[20]</sup> Other dietary factors that may increase prostate cancer risk include low intake of vitamin E (Vitamin E is found in green, leafy vegetables), omega-3 fatty acids (found in fatty fishes like salmon), and the mineral selenium. A study in 2007 cast doubt on the effectiveness of lycopene (found in tomatoes) in reducing the risk of prostate cancer.<sup>[21]</sup> Lower blood levels of vitamin D also may increase the risk of developing prostate cancer. This may be linked to lower exposure to ultraviolet (UV) light, since UV light exposure can increase vitamin D in the body.<sup>[22]</sup>

There are also some links between prostate cancer and medications, medical procedures, and medical conditions. Daily use of anti-inflammatory medicines such as aspirin, ibuprofen, or naproxen may decrease prostate cancer risk.<sup>[23]</sup> Use of the cholesterol-lowering drugs known as the statins may also decrease prostate cancer risk.<sup>[24]</sup> More frequent ejaculation also may decrease a man's risk of prostate cancer. One study showed that men who ejaculated five times a week in their 20s had a decreased rate of prostate cancer, though others have shown no benefit.<sup>[25][26]</sup> Infection or inflammation of the prostate (prostatitis) may increase the chance for prostate cancer. In particular, infection with the sexually transmitted infections chlamydia, gonorrhea, or syphilis seems to increase risk.<sup>[27]</sup> Finally, obesity<sup>[28]</sup> and elevated blood levels of testosterone<sup>[29]</sup> may increase the risk for prostate cancer.

Research released in May 2007, found that US war veterans who had been exposed to Agent Orange had a 48% increased risk of prostate cancer recurrence following surgery.<sup>[30]</sup>

Prostate cancer risk can be decreased by modifying known risk factors for prostate cancer, such as decreasing intake of animal fat.<sup>[31]</sup>

One research study, by the Cancer Council Victoria, has shown that men who report that they regularly ("more than five times per week") masturbate have up to one third fewer occurrences of prostate cancer.<sup>[32]</sup> The researchers hypothesize that this could be because regular ejaculation reduces the buildup of carcinogenic deposits which could damage the cells lining the prostate. The researchers also speculated that frequent ejaculation may cause the prostate to mature fully, making it less susceptible to carcinogens. It is also possible that there is another factor (such as hormone levels) that is a common cause of both a reduced susceptibility to prostate cancer and a tendency toward frequent masturbation. There is also some evidence that frequent sexual intercourse is associated with reduced risk of prostate cancer, although contrarily the risks associated with STDs have been shown to increase the risk of prostate cancer<sup>[33]</sup>. Once the lining of the prostate is affected with cancer, the only known treatments are surgery and radiation therapy. Both may limit the ability to have erections afterward.

## Prevention

Several medications and vitamins may also help prevent prostate cancer. Two dietary supplements, vitamin E and selenium, may help prevent prostate cancer when taken daily. Estrogens from fermented soybeans and other plant sources (called phytoestrogens) may also help prevent prostate cancer.<sup>[34]</sup> The selective estrogen receptor modulator drug toremifene has shown promise in early trials.<sup>[35][36]</sup> Two medications which block the conversion of testosterone to dihydrotestosterone, finasteride<sup>[37]</sup> and dutasteride,<sup>[38]</sup> have also shown some promise. As of 2006 the use of these medications for primary prevention is still in the testing phase, and they are not widely used for this purpose. The problem with these medications is that they may preferentially block the development of lower-grade prostate tumors, leading to a relatively greater chance of higher grade cancers, and negating any overall survival improvement. Green tea may be protective (due to its polyphenol

content), though the data is mixed.<sup>[39][40]</sup> A 2006 study of green tea derivatives demonstrated promising prostate cancer prevention in patients at high risk for the disease.<sup>[41]</sup> In 2003, an Australian research team led by Graham Giles of The Cancer Council Australia concluded that frequent masturbation by males appears to help prevent the development of prostate cancer.<sup>[42]</sup> Recent research published in the Journal of the National Cancer Institute suggests that taking multivitamins more than seven times a week can increase the risks of contracting the disease.<sup>[43]</sup> This research was unable to highlight the exact vitamins responsible for this increase (almost double), although they suggest that vitamin A, vitamin E and beta-carotene may lie at its heart. It is advised that those taking multivitamins never exceed the stated daily dose on the label. Scientists recommend a healthy, well balanced diet rich in fiber, and to reduce intake of meat. A 2007 study published in the Journal of the National Cancer Institute found that men eating cauliflower, broccoli, or one of the other cruciferous vegetables, more than once a week were 40% less likely to develop prostate cancer than men who rarely ate those vegetables.<sup>[44]</sup> Scientists believe the reason for this phenomenon has to do with a phytochemical called Diindolylmethane in these vegetables that has anti-androgenic and immune modulating properties. This compound is currently under investigation by the National Cancer Institute as a natural therapeutic for prostate cancer.

## Capsaicin

Capsaicin, the chemical found in peppers, has been shown to cause 80% of cancerous prostate cells to undergo apoptosis in mice. For prostate cancer cells whose growth is dependent upon testosterone, Capsaicin curbed the proliferation of such cells by freezing the cells in a non-proliferate state, and cancerous prostate cells that are androgen independent "suicided" as well.<sup>[45]</sup>

"Capsaicin had a profound anti-proliferative effect on human prostate cancer cells in culture," said Sören Lehmann, M.D., Ph.D., visiting scientist at the Cedars-Sinai Medical Center and the UCLA School of Medicine. "It also dramatically slowed the development of prostate tumors formed by those human cell lines grown in mouse models."<sup>[46]</sup>

Peppers which rank higher on the Scoville scale and thus have a higher piquancy contain a higher amount of Capsaicin. Habaneros, for example, have a Scoville rating of over 300,000, while red chili peppers have a rating of 5,000. While the UCLA and Samuel Oschin Comprehensive Cancer Institute studies show promising implications, it is not yet confirmed the same effects can be duplicated in men.<sup>[47]</sup>

## Screening

Prostate cancer screening is an attempt to find unsuspected cancers. Screening tests may lead to more specific follow-up tests such as a biopsy, where small pieces of the prostate are removed for closer study. As of 2006 prostate cancer screening options include the digital rectal exam and the prostate specific antigen (PSA) blood test. Screening for prostate cancer is controversial because it is not clear if the benefits of screening outweigh the risks of follow-up diagnostic tests and cancer treatments.

Prostate cancer is a slow-growing cancer, very common among older men. In fact, most prostate cancers never grow to the point where they cause symptoms, and most men with prostate cancer die of other causes before prostate cancer has an impact on their lives. The PSA screening test may detect these small cancers that would never become life threatening. Doing the PSA test in these men may lead to overdiagnosis, including additional testing and treatment. Follow-up tests, such as prostate biopsy, may cause pain, bleeding and infection. Prostate cancer treatments may cause urinary incontinence and erectile dysfunction. Therefore, it is essential that the risks and benefits of diagnostic procedures and treatment be carefully considered before PSA screening.

Prostate cancer screening generally begins after age 50, but this can vary due to ethnic backgrounds. An example of this is African American men, who have the highest overall rate of prostate cancer.<sup>[48]</sup> It has thus been recommended to begin screening checks at age 35,<sup>[49]</sup> especially for African American males who have a strong family history of prostate cancer.<sup>[50]</sup> The American Academy of Family Physicians and American College of Physicians recommend the physician discuss the risks and benefits of screening and decide based on individual patient preference.<sup>[51]</sup> Although there is no officially recommended cutoff, many health care providers stop monitoring PSA in men who are older than 75 years old because of concern that prostate cancer therapy may do more harm than good as age progresses and life expectancy decreases.

## Digital rectal examination

Digital rectal examination (DRE) is a procedure where the examiner inserts a gloved, lubricated finger into the rectum to check the size, shape, and texture of the prostate. Areas which are irregular, hard or lumpy need further evaluation, since they may contain cancer. Although the DRE only evaluates the back of the prostate, 85% of prostate cancers arise in this part of the prostate. Prostate cancer which can be felt on DRE is generally more advanced.<sup>[52]</sup> The use of DRE has never been shown to prevent prostate cancer deaths when used as the only screening test.<sup>[53]</sup>

## Prostate specific antigen

The PSA test measures the blood level of prostate-specific antigen, an enzyme produced by the prostate. Specifically, PSA is a serine protease similar to kallikrein. Its normal function is to liquify gelatinous semen after ejaculation, allowing spermatazoa to more easily navigate through the uterine cervix.

PSA levels under 4 ng/mL (nanograms per milliliter) are generally considered normal, however in individuals below the age of 50 sometimes a cutoff of 2.5 is used for the upper limit of normal, while levels over 4 ng/mL are considered abnormal (although in men over 65 levels up to 6.5 ng/mL may be acceptable, depending upon each laboratory's reference ranges). PSA levels between 4 and 10 ng/mL indicate a risk of prostate cancer higher than normal, but the risk does not seem to rise within this six-point range. When the PSA level is above 10 ng/mL, the association with cancer becomes stronger. However, PSA is not a perfect test. Some men with prostate cancer do not have an elevated PSA, and most men with an elevated PSA do not have prostate cancer.

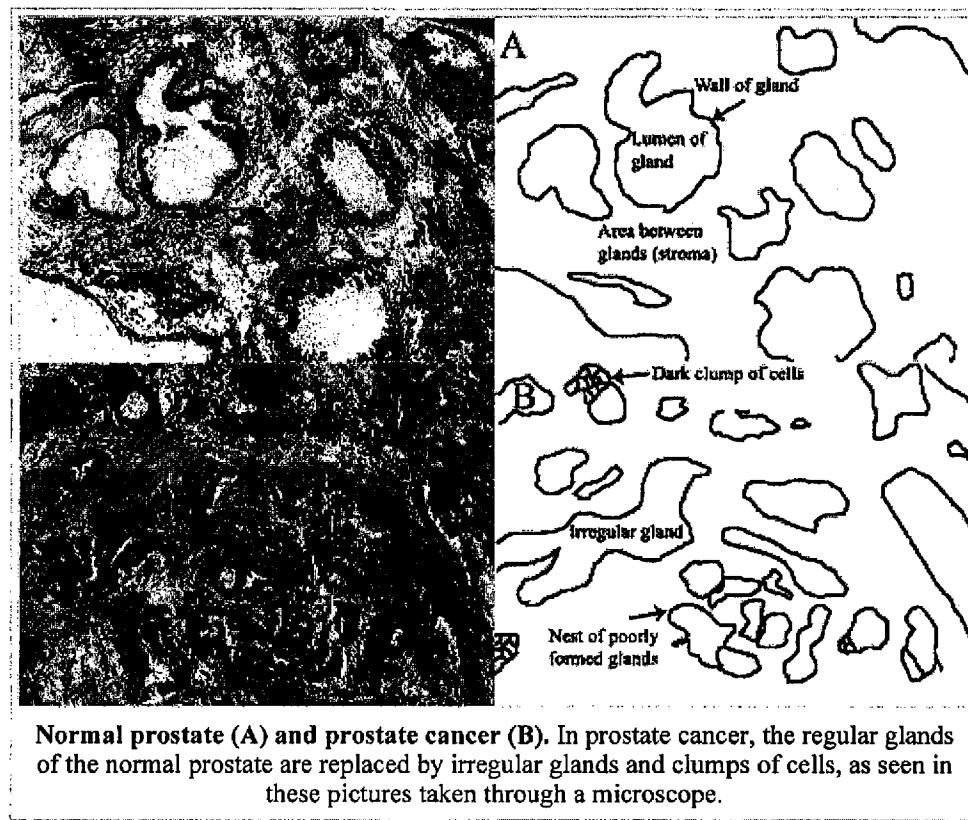
PSA levels can change for many reasons other than cancer. Two common causes of high PSA levels are enlargement of the prostate (benign prostatic hypertrophy (BPH)) and infection in the prostate (prostatitis). It can also be raised for 24 hours after ejaculation and several days after catheterization. PSA levels are lowered in men who use medications used to treat BPH or baldness. These medications, finasteride (marketed as Proscar or Propecia) and dutasteride (marketed as Avodart), may decrease the PSA levels by 50% or more.

Several other ways of evaluating the PSA have been developed to avoid the shortcomings of simple PSA screening. The use of age-specific reference ranges improves the sensitivity and specificity of the test. The rate of rise of the PSA over time, called the PSA velocity, has been used to evaluate men with PSA levels between 4 and 10 ng/ml, but as of 2006, it has not proven to be an effective screening test.<sup>[54]</sup> Comparing the PSA level with the size of the prostate, as measured by ultrasound or magnetic resonance imaging, has also been studied. This comparison, called PSA density, is both costly and, as of 2006, has not proven to be an effective screening test.<sup>[55]</sup> PSA in the blood may either be free or bound to other proteins. Measuring the amount of PSA which is free or bound may provide additional screening information, but as of 2006, questions regarding the usefulness of these measurements limit their widespread use.<sup>[56][57]</sup>

## Diagnosis

When a man has symptoms of prostate cancer, or a screening test indicates an increased risk for cancer, more invasive evaluation is offered.

The only test which can fully confirm the diagnosis of prostate cancer is a biopsy, the removal of small pieces of the prostate for microscopic examination. However, prior to a biopsy, several other tools may be used to gather more information about the prostate and the urinary tract. Cystoscopy shows the urinary tract from inside the bladder, using a thin, flexible camera tube inserted down the urethra. Transrectal ultrasonography creates a picture of the prostate using sound waves from a probe in the rectum.



**Normal prostate (A) and prostate cancer (B).** In prostate cancer, the regular glands of the normal prostate are replaced by irregular glands and clumps of cells, as seen in these pictures taken through a microscope.

## Biopsy

If cancer is suspected, a biopsy is offered. During a biopsy a urologist obtains tissue samples from the prostate via the rectum. A biopsy gun inserts and removes special hollow-core needles (usually three to six on each side of the prostate) in less than a second. Prostate biopsies are routinely done on an outpatient basis and rarely require hospitalization. Fifty-five percent of men report discomfort during prostate biopsy.<sup>[58]</sup>

### Gleason score

The tissue samples are then examined under a microscope to determine whether cancer cells are present, and to evaluate the microscopic features (or Gleason score) of any cancer found.

### Tumor markers

Tissue samples can be stained for the presence of PSA and other tumor markers in order to determine the origin of malignant cells that have metastasized.<sup>[59]</sup>

### New tests being investigated

Currently, an active area of research involves non-invasive methods of prostate tumor detection. Adenoviruses modified to transfect tumor cells with harmless yet distinct genes (such as luciferase) have

proven capable of early detection. So far, though, this area of research has only been tested in animal and LNCaP models.<sup>[60]</sup>

### PCA3

Another potential non-invasive methods of early prostate tumor detection is through a molecular test that detects the presence of cell-associated PCA3 mRNA in urine. PCA3 mRNA is expressed almost exclusively by prostate cells and has been shown to be highly over-expressed in prostate cancer cells. PCA3 is not a replacement for PSA but an additional tool to help decide if, in men suspected of having prostate cancer, a biopsy is really needed. The higher the expression of PCA3 in urine, the greater the likelihood of a positive biopsy, i.e. the presence of cancer cells in the prostate.

### Early prostate cancer

It was reported in April 2007 that a new blood test for early prostate cancer antigen-2 (EPCA-2) is being researched that may alert men if they have prostate cancer and how aggressive it will be.<sup>[61][62]</sup>

### Staging

An important part of evaluating prostate cancer is determining the stage, or how far the cancer has spread. Knowing the stage helps define prognosis and is useful when selecting therapies. The most common system is the four-stage TNM system (abbreviated from Tumor/Nodes/Metastases). Its components include the size of the tumor, the number of involved lymph nodes, and the presence of any other metastases.

The most important distinction made by any staging system is whether or not the cancer is still confined to the prostate. In the TNM system, clinical T1 and T2 cancers are found only in the prostate, while T3 and T4 cancers have spread elsewhere. Several tests can be used to look for evidence of spread. These include computed tomography to evaluate spread within the pelvis, bone scans to look for spread to the bones, and endorectal coil magnetic resonance imaging to closely evaluate the prostatic capsule and the seminal vesicles. Bone scans should reveal osteoblastic appearance due to *increased* bone density in the areas of bone metastasis - opposite to what is found in many other cancers that metastasize.

After a prostate biopsy, a pathologist looks at the samples under a microscope. If cancer is present, the pathologist reports the grade of the tumor. The grade tells how much the tumor tissue differs from normal prostate tissue and suggests how fast the tumor is likely to grow. The Gleason system is used to grade prostate tumors from 2 to 10, where a Gleason score of 10 indicates the most abnormalities. The pathologist assigns a number from 1 to 5 for the most common pattern observed under the microscope, then does the same for the second most common pattern. The sum of these two numbers is the Gleason score. The Whitmore-Jewett stage is another method sometimes used. Proper grading of the tumor is critical, since the grade of the tumor is one of the major factors used to determine the treatment recommendation.

### Risk assessment

Many prostate cancers are not destined to be lethal, and most men will ultimately die from causes other than of the disease. Decisions about treatment type and timing may therefore be informed by an estimation of the risk that the tumor will ultimately recur after treatment and/or progress to metastases and mortality. Several tools are available to help predict outcomes such as pathologic stage and recurrence after surgery or radiation therapy. Most combine stage, grade, and PSA level, and some also add the number or percent of biopsy cores positive, age, and/or other information.

The D'Amico classification stratifies men to low, intermediate, or high risk based on stage, grade, and PSA. It is used widely in clinical practice and research settings. The major downside to the 3-level system is that it does not account for multiple adverse parameters (e.g., high Gleason score *and* high PSA) in stratifying patients.

The Partin tables predict pathologic outcomes (margin status, extraprostatic extension, and seminal vesicle invasion) based on the same 3 variables, and are published as lookup tables.

The Kattan nomograms predict recurrence after surgery and/or radiation therapy, based on data available either at time of diagnosis or after surgery. The nomograms can be calculated using paper graphs, or using software available on a website or for handheld computers. The Kattan score represents the likelihood of remaining free of disease at a given time interval following treatment.

The UCSF Cancer of the Prostate Risk Assessment (CAPRA) score predicts both pathologic status and recurrence after surgery. It offers comparable accuracy as the Kattan preoperative nomogram, and can be calculated without paper tables or a calculator. Points are assigned based on PSA, Grade, stage, age, and percent of cores positive; the sum yields a 0–10 score, with every 2 points representing roughly a doubling of risk of recurrence. The CAPRA score was derived from community-based data in the CaPSURE database.

## Treatment

Treatment for prostate cancer may involve watchful waiting, surgery, radiation therapy, High Intensity Focused Ultrasound (HIFU), chemotherapy, cryosurgery, hormonal therapy, or some combination. Which option is best depends on the stage of the disease, the Gleason score, and the PSA level. Other important factors are the man's age, his general health, and his feelings about potential treatments and their possible side effects. Because all treatments can have significant side effects, such as erectile dysfunction and urinary incontinence, treatment discussions often focus on balancing the goals of therapy with the risks of lifestyle alterations.

The selection of treatment options may be a complex decision involving many factors. For example, radical prostatectomy after primary radiation failure is a very technically challenging surgery and may not be an option.<sup>[63]</sup> This may enter into the treatment decision.

If the cancer has spread beyond the prostate, treatment options significantly change, so most doctors who treat prostate cancer use a variety of nomograms to predict the probability of spread. Treatment by watchful waiting, HIFU, radiation therapy, cryosurgery, and surgery are generally offered to men whose cancer remains within the prostate. Hormonal therapy and chemotherapy are often reserved for disease which has spread beyond the prostate. However, there are exceptions: radiation therapy may be used for some advanced tumors, and hormonal therapy is used for some early stage tumors. Cryotherapy, hormonal therapy, and chemotherapy may also be offered if initial treatment fails and the cancer progresses.

### Watchful waiting and active surveillance

Watchful waiting, also called "active surveillance," refers to observation and regular monitoring without invasive treatment. Watchful waiting is often used when an early stage, slow-growing prostate cancer is found in an older man. Watchful waiting may also be suggested when the risks of surgery, radiation therapy, or hormonal therapy outweigh the possible benefits. Other treatments can be started if symptoms develop, or if there are signs that the cancer growth is accelerating (e.g., rapidly rising PSA, increase in Gleason score on repeat biopsy, etc.). Most men who choose watchful waiting for early stage tumors eventually have signs of tumor progression, and they may need to begin treatment within three years.<sup>[64]</sup> Although men who choose

watchful waiting avoid the risks of surgery and radiation, the risk of metastasis (spread of the cancer) may be increased. For younger men, a trial of active surveillance may not mean avoiding treatment altogether, but may reasonably allow a delay of a few years or more, during which time the quality of life impact of active treatment can be avoided. Published data to date suggest that carefully selected men will not miss a window for cure with this approach. Additional health problems that develop with advancing age during the observation period can also make it harder to undergo surgery and radiation therapy.

Clinically insignificant prostate tumors are often found by accident when a doctor incorrectly orders a biopsy not following the recommended guidelines (abnormal DRE and elevated PSA). The urologist must check that the PSA is not elevated for other reasons, Prostatitis, etc. An annual biopsy is often recommended by a urologist for a patient who has selected watchful waiting when the tumor is clinically insignificant (no abnormal DRE or PSA). The tumors tiny size can be monitored this way and the patient can decide to have surgery only if the tumor enlarges which may take many years or never.

## Surgery

Surgical removal of the prostate, or prostatectomy, is a common treatment either for early stage prostate cancer, or for cancer which has failed to respond to radiation therapy. The most common type is radical retropubic prostatectomy, when the surgeon removes the prostate through an abdominal incision. Another type is radical perineal prostatectomy, when the surgeon removes the prostate through an incision in the perineum, the skin between the scrotum and anus. Radical prostatectomy can also be performed laparoscopically, through a series of small (1cm) incisions in the abdomen, with or without the assistance of a surgical robot.

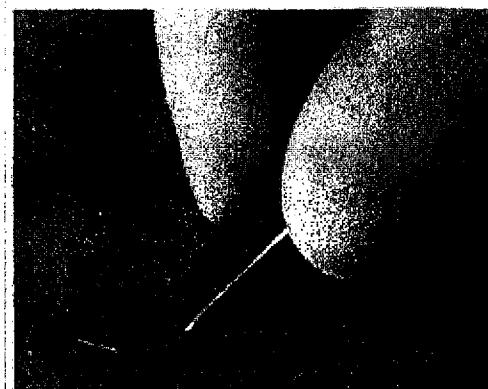
Radical prostatectomy is effective for tumors which have not spread beyond the prostate.<sup>[65]</sup> Cure rates depend on risk factors such as PSA level and Gleason grade. However, it may cause nerve damage that significantly alters the quality of life of the prostate cancer survivor. The most common serious complications are loss of urinary control and impotence. Reported rates of both complications vary widely depending on how they are assessed, by whom, and how long after surgery, as well as the setting (e.g., academic series vs. community-based or population-based data). Although penile sensation and the ability to achieve orgasm usually remain intact, erection and ejaculation are often impaired. Medications such as sildenafil (Viagra), tadalafil (Cialis), or vardenafil (Levitra) may restore some degree of potency. For most men with organ-confined disease, a more limited "nerve-sparing" technique may help avoid urinary incontinence and impotence.<sup>[66]</sup>

Radical prostatectomy has traditionally been used alone when the cancer is small. In the event of positive margins or locally advanced disease found on pathology, adjuvant radiation therapy may offer improved survival. Surgery may also be offered when a cancer is not responding to radiation therapy. However, because radiation therapy causes tissue changes, prostatectomy after radiation has a higher risk of complications.

Transurethral resection of the prostate, commonly called a "TURP," is a surgical procedure performed when the tube from the bladder to the penis (urethra) is blocked by prostate enlargement. TURP is generally for benign disease and is not meant as definitive treatment for prostate cancer. During a TURP, a small tube (cystoscope) is placed into the penis and the blocking prostate is cut away.

In metastatic disease, where cancer has spread beyond the prostate, removal of the testicles (called orchectomy) may be done to decrease testosterone levels and control cancer growth. (See hormonal therapy, below).

## Radiation therapy



**Brachytherapy** for prostate cancer is administered using "seeds," small radioactive rods implanted directly into the tumor.

Radiation therapy, also known as radiotherapy, uses ionizing radiation to kill prostate cancer cells. When absorbed in tissue, Ionizing radiation such as Gamma and x-rays damage the DNA in cells, which increases the probability of apoptosis (cell death). Two different kinds of radiation therapy are used in prostate cancer treatment: external beam radiation therapy and brachytherapy.

External beam radiation therapy uses a linear accelerator to produce high-energy x-rays which are directed in a beam towards the prostate. A technique called Intensity Modulated Radiation Therapy (IMRT) may be used to adjust the radiation beam to conform with the shape of the tumor, allowing higher doses to be given to the prostate and seminal vesicles with less damage to the bladder and rectum. External beam radiation therapy is generally given over several weeks, with daily visits to a radiation therapy center. New types of radiation therapy may have fewer side

effects than traditional treatment, one of these is Tomotherapy.

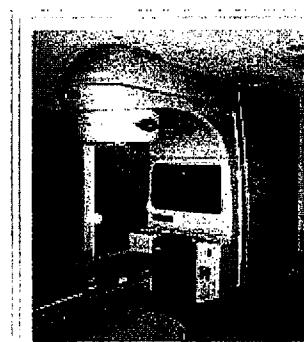
Permanent implant brachytherapy is a popular treatment choice for patients with low to intermediate risk features, can be performed on an outpatient basis, and is associated with good 10-year outcomes with relatively low morbidity<sup>[67]</sup> It involves the placement of about 100 small "seeds" containing radioactive material (such as iodine-125 or palladium-103) with a needle through the skin of the perineum directly into the tumor while under spinal or general anesthetic. These seeds emit lower-energy X-rays which are only able to travel a short distance. Although the seeds eventually become inert, they remain in the prostate permanently. The risk of exposure to others from men with implanted seeds is generally accepted to be insignificant.<sup>[68]</sup>

Radiation therapy is commonly used in prostate cancer treatment. It may be used instead of surgery for early cancers, and it may also be used in advanced stages of prostate cancer to treat painful bone metastases. Radiation treatments also can be combined with hormonal therapy for intermediate risk disease, when radiation therapy alone is less likely to cure the cancer. Some radiation oncologists combine external beam radiation and brachytherapy for intermediate to high risk situations. One study found that the combination of six months of androgen suppressive therapy combined with external beam radiation had improved survival compared to radiation alone in patients with localized prostate cancer.<sup>[69]</sup> Others use a "triple modality" combination of external beam radiation therapy, brachytherapy, and hormonal therapy.

Less common applications for radiotherapy are when cancer is compressing the spinal cord, or sometimes after surgery, such as when cancer is found in the seminal vesicles, in the lymph nodes, outside the prostate capsule, or at the margins of the biopsy.

Radiation therapy is often offered to men whose medical problems make surgery more risky. Radiation therapy appears to cure small tumors that are confined to the prostate just about as well as surgery. However, as of 2006 some issues remain unresolved, such as whether radiation should be given to the rest of the pelvis, how much the absorbed dose should be, and whether hormonal therapy should be given at the same time.

Side effects of radiation therapy might occur after a few weeks into treatment. Both types of radiation therapy



External beam radiation therapy for prostate cancer is delivered by a linear accelerator, such as this one.

may cause diarrhea and rectal bleeding due to radiation proctitis, as well as urinary incontinence and impotence. Symptoms tend to improve over time.<sup>[70]</sup> Men who have undergone external beam radiation therapy will have a higher risk of later developing colon cancer and bladder cancer.<sup>[71]</sup>

## Cryosurgery

Cryosurgery is another method of treating prostate cancer. It is less invasive than radical prostatectomy, and general anesthesia is less commonly used. Under ultrasound guidance, a method invented by Dr. Gary Onik,<sup>[72]</sup> metal rods are inserted through the skin of the perineum into the prostate. Highly purified Argon gas is used to cool the rods, freezing the surrounding tissue at  $-196^{\circ}\text{C}$  ( $-320^{\circ}\text{F}$ ). As the water within the prostate cells freeze, the cells die. The urethra is protected from freezing by a catheter filled with warm liquid. Cryosurgery generally causes fewer problems with urinary control than other treatments, but impotence occurs up to ninety percent of the time. When used as the initial treatment for prostate cancer and in the hands of an experienced cryosurgeon, cryosurgery has a 10 year biochemical disease free rate superior to all other treatments including radical prostatectomy and any form of radiation.<sup>[73]</sup> Cryosurgery has also been demonstrated to be superior to radical prostatectomy for recurrent cancer following radiation therapy.

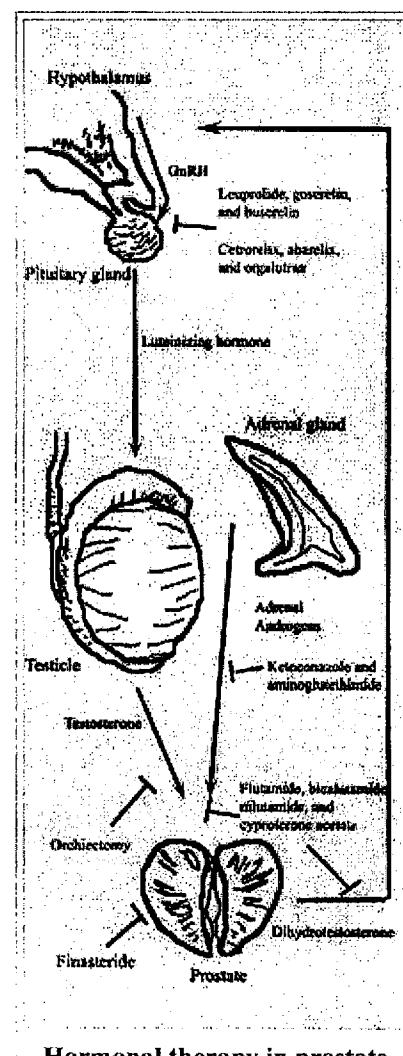
## Hormonal therapy

Hormonal therapy uses medications or surgery to block prostate cancer cells from getting dihydrotestosterone (DHT), a hormone produced in the prostate and required for the growth and spread of most prostate cancer cells. Blocking DHT often causes prostate cancer to stop growing and even shrink. However, hormonal therapy rarely cures prostate cancer because cancers which initially respond to hormonal therapy typically become resistant after one to two years. Hormonal therapy is therefore usually used when cancer has spread from the prostate. It may also be given to certain men undergoing radiation therapy or surgery to help prevent return of their cancer.<sup>[74]</sup>

Hormonal therapy for prostate cancer targets the pathways the body uses to produce DHT. A feedback loop involving the testicles, the hypothalamus, and the pituitary, adrenal, and prostate glands controls the blood levels of DHT. First, low blood levels of DHT stimulate the hypothalamus to produce gonadotropin releasing hormone (GnRH). GnRH then stimulates the pituitary gland to produce luteinizing hormone (LH), and LH stimulates the testicles to produce testosterone. Finally, testosterone from the testicles and dehydroepiandrosterone from the adrenal glands stimulate the prostate to produce more DHT. Hormonal therapy can decrease levels of DHT by interrupting this pathway at any point.

There are several forms of hormonal therapy:

- Orchiectomy is surgery to remove the testicles. Because the testicles make most of the body's testosterone, after orchiectomy testosterone levels drop. Now the prostate not only lacks the testosterone stimulus to produce DHT, but also it does not have enough testosterone to transform into DHT.
- Antiandrogens are medications such as flutamide, bicalutamide, nilutamide, and cyproterone acetate which directly block the



**Hormonal therapy in prostate cancer.** Diagram shows the different organs (purple text).

actions of testosterone and DHT within prostate cancer cells.

- Medications which block the production of adrenal androgens such as DHEA include ketoconazole and aminoglutethimide. Because the adrenal glands only make about 5% of the body's androgens, these medications are generally used only in combination with other methods that can block the 95% of androgens made by the testicles. These combined methods are called total androgen blockade (TAB). TAB can also be achieved using antiandrogens.
- GnRH action can be interrupted in one of two ways. GnRH antagonists suppress the production of LH directly, while GnRH agonists suppress LH through the process of downregulation after an initial stimulation effect. Abarelix is an example of a GnRH antagonist, while the GnRH agonists include leuprorelin, goserelin, triptorelin, and buserelin. Initially, GnRH agonists *increase* the production of LH. However, because the constant supply of the medication does not match the body's natural production rhythm, production of both LH and GnRH decreases after a few weeks.<sup>[75]</sup>

hormones (*black text and arrows*), and treatments (*red text and arrows*) important in hormonal therapy.

As of 2006 the most successful hormonal treatments are orchiectomy and GnRH agonists. Despite their higher cost, GnRH agonists are often chosen over orchiectomy for cosmetic and emotional reasons. Eventually, total androgen blockade may prove to be better than orchiectomy or GnRH agonists used alone.

Each treatment has disadvantages which limit its use in certain circumstances. Although orchiectomy is a low-risk surgery, the psychological impact of removing the testicles can be significant. The loss of testosterone also causes hot flashes, weight gain, loss of libido, enlargement of the breasts (gynecomastia), impotence and osteoporosis. GnRH agonists eventually cause the same side effects as orchiectomy but may cause worse symptoms at the beginning of treatment. When GnRH agonists are first used, testosterone surges can lead to increased bone pain from metastatic cancer, so antiandrogens or abarelix are often added to blunt these side effects. Estrogens are not commonly used because they increase the risk for cardiovascular disease and blood clots. The antiandrogens do not generally cause impotence and usually cause less loss of bone and muscle mass. Ketoconazole can cause liver damage with prolonged use, and aminoglutethimide can cause skin rashes.

## Palliative care

Palliative care for advanced stage prostate cancer focuses on extending life and relieving the symptoms of metastatic disease. Chemotherapy may be offered to slow disease progression and postpone symptoms. The most commonly used regimen combines the chemotherapeutic drug docetaxel with a corticosteroid such as prednisone.<sup>[76]</sup> Bisphosphonates such as zoledronic acid have been shown to delay skeletal complications such as fractures or the need for radiation therapy in patients with hormone-refractory metastatic prostate cancer.<sup>[77]</sup>

Bone pain due to metastatic disease is treated with opioid pain relievers such as morphine and oxycodone. External beam radiation therapy directed at bone metastases may provide pain relief. Injections of certain radioisotopes, such as strontium-89, phosphorus-32, or samarium-153, also target bone metastases and may help relieve pain.

## High Intensity Focused Ultrasound (HIFU)

HIFU for prostate cancer utilizes high intensity focused ultrasound (HIFU) to ablate/destroy the tissue of the prostate. During the HIFU procedure, sound waves are used to heat the prostate tissue thus destroying the cancerous cells. Essentially, ultrasonic waves are precisely focused on specific areas of the prostate to eliminate the prostate cancer with minimal risks of affecting other tissue or organs. Temperatures at the focal point of the sound waves can exceed 100°C.<sup>[78]</sup> In lay terms, the HIFU technology is similar to using a

magnifying glass to burn a piece of paper by focusing sunlight at a small precise point on the sheet. The ability to focus the ultrasonic waves leads to a relatively low occurrence of both incontinence and impotence. (0.6% and 0-20%, respectively)<sup>[79]</sup> According to international studies, when compared to other procedures, HIFU has a high success rate with a reduced risk of side effects. Studies using the Sonablate 500 HIFU machine have shown that 94% of patients with a pretreatment PSA (Prostate Specific Antigen) of less than 10 g/ml were cancer-free after three years.<sup>[80]</sup> However, many studies of HIFU were performed by manufacturers of HIFU devices, or members of manufacturers' advisory panels.<sup>[81]</sup>

HIFU was first used in the 1940's and 1950's in efforts to destroy tumors in the central nervous system. Since then, HIFU has been shown to be effective at destroying malignant tissue in the brain, prostate, spleen, liver, kidney, breast, and bone.<sup>[82]</sup> Today, the HIFU procedure for prostate cancer is performed using a transrectal probe. This procedure has been performed for over ten years and is currently approved for use in Japan, Europe, Canada, and parts of Central and South America.

Although not yet approved for use in the United States, many patients have received the HIFU procedure at facilities in Canada, and Central and South America. Currently, therapy is available using the Sonablate 500 or the Ablatherm. The Sonablate 500 is designed by Focus Surgery of Indianapolis, Indiana and is used in international HIFU centers around the world.

## Prognosis

Prostate cancer rates are higher and prognosis poorer in developed countries than the rest of the world. Many of the risk factors for prostate cancer are more prevalent in the developed world, including longer life expectancy and diets high in red meat and dairy products.<sup>[83]</sup> Also, where there is more access to screening programs, there is a higher detection rate. Prostate cancer is the ninth most common cancer in the world, but is the number one non-skin cancer in United States men. Prostate cancer affected eighteen percent of American men and caused death in three percent in 2005.<sup>[84]</sup> In Japan, death from prostate cancer was one-fifth to one-half the rates in the United States and Europe in the 1990s.<sup>[85]</sup> In India in the 1990s, half of the people with prostate cancer confined to the prostate died within ten years.<sup>[86]</sup> African-American men have 50–60 times more prostate cancer and prostate cancer deaths than men in Shanghai, China.<sup>[87]</sup> In Nigeria, two percent of men develop prostate cancer and 64% of them are dead after two years.<sup>[88]</sup>

In patients who undergo treatment, the most important clinical prognostic indicators of disease outcome are stage, pre-therapy PSA level and Gleason score. In general, the higher the grade and the stage, the poorer the prognosis. Nomograms can be used to calculate the estimated risk of the individual patient. The predictions are based on the experience of large groups of patients suffering from cancers at various stages.<sup>[89]</sup>

## Progression

In 1941, Charles Huggins reported that androgen ablation therapy causes regression of primary and metastatic androgen-dependent prostate cancer.<sup>[90]</sup> However, it is now known that 80–90% of prostate cancer patients develop androgen-independent tumors 12–33 months after androgen ablation therapy, leading to a median overall survival of 23–37 months from the time of initiation of androgen ablation therapy.<sup>[91]</sup> The actual mechanism contributes to the progression of prostate cancer is not clear and may vary between individual patient. A few possible mechanisms have been proposed.<sup>[92]</sup> Scientists have established a few prostate cancer cell lines to investigate the mechanism involved in the progression of prostate cancer. LNCaP, PC-3, and DU-145 are commonly used prostate cancer cell lines. The LNCaP cancer cell line was established from a human lymph node metastatic lesion of prostatic adenocarcinoma. PC-3 and DU-145 cells were established from human prostatic adenocarcinoma metastatic to bone and to brain, respectively. LNCaP cells express androgen

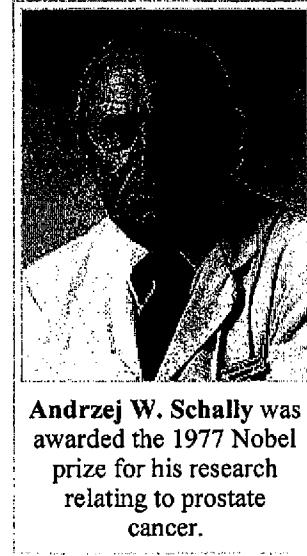
receptor (AR), however, PC-3 and DU-145 cells express very little or no AR. AR, an androgen-activated transcription factor, belongs to the steroid nuclear receptor family. Development of the prostate is dependent on androgen signaling mediated through AR, and AR is also important during the development of prostate cancer. The proliferation of LNCaP cells is androgen-dependent but the proliferation of PC-3 and DU-145 cells is androgen-insensitive. Elevation of AR expression is often observed in advanced prostate tumors in patients.<sup>[93][94]</sup> Some androgen-independent LNCaP sublines have been developed from the ATCC androgen-dependent LNCaP cells after androgen deprivation for study of prostate cancer progression. These androgen-independent LNCaP cells have elevated AR expression and express prostate specific antigen upon androgen treatment. Androgens paradoxically inhibit the proliferation of these androgen-independent prostate cancer cells.<sup>[95][96][97]</sup> Androgen at a concentration of 10-fold higher than the physiological concentration has also been shown to cause growth suppression and reversion of androgen-independent prostate cancer xenografts or androgen-independent prostate tumors derived *in vivo* model to an androgen-stimulated phenotype in athymic mice.<sup>[98][99]</sup> These observations suggest the possibility to use androgen to treat the development of relapsed androgen-independent prostate tumors in patients. Oral infusion of green tea polyphenols, a potential alternative therapy for prostate cancer by natural compounds, has been shown to inhibit the development, progression, and metastasis as well as autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which spontaneously develops prostate cancer.<sup>[100]</sup>

## History

Although the prostate was first described by Venetian anatomist Niccolò Massa in 1536, and illustrated by Flemish anatomist Andreas Vesalius in 1538, prostate cancer was not identified until 1853.<sup>[101]</sup> Prostate cancer was initially considered a rare disease, probably because of shorter life expectancies and poorer detection methods in the 19th century. The first treatments of prostate cancer were surgeries to relieve urinary obstruction.<sup>[102]</sup> Removal of the entire gland (radical perineal prostatectomy) was first performed in 1904 by Hugh H. Young at Johns Hopkins Hospital.<sup>[103]</sup> Surgical removal of the testes (orchietomy) to treat prostate cancer was first performed in the 1890s, but with limited success. Transurethral resection of the prostate (TURP) replaced radical prostatectomy for symptomatic relief of obstruction in the middle of the 20th century because it could better preserve penile erectile function. Radical retropubic prostatectomy was developed in 1983 by Patrick Walsh.<sup>[104]</sup> This surgical approach allowed for removal of the prostate and lymph nodes with maintenance of penile function.

In 1941 Charles B. Huggins published studies in which he used estrogen to oppose testosterone production in men with metastatic prostate cancer. This discovery of "chemical castration" won Huggins the 1966 Nobel Prize in Physiology or Medicine.<sup>[105]</sup> The role of the hormone GnRH in reproduction was determined by Andrzej W. Schally and Roger Guillemin, who both won the 1977 Nobel Prize in Physiology or Medicine for this work. Receptor agonists, such as leuprolide and goserelin, were subsequently developed and used to treat prostate cancer.<sup>[106][107]</sup>

Radiation therapy for prostate cancer was first developed in the early 20th century and initially consisted of intraprostatic radium implants. External beam radiation became more popular as stronger radiation sources became available in the middle of the 20th century. Brachytherapy with implanted seeds was first described in 1983.<sup>[108]</sup> Systemic chemotherapy for prostate cancer was first studied in the 1970s. The initial regimen of cyclophosphamide and 5-fluorouracil was quickly joined by multiple regimens using a host of other systemic chemotherapy drugs.<sup>[109]</sup>



Andrzej W. Schally was awarded the 1977 Nobel prize for his research relating to prostate cancer.

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## See also

- Testosterone

## External links

- Prostate cancer at the Open Directory Project
- Prostate Cancer Research Foundation of Canada
- Malecare Prostate Cancer Support
- Prostate Cancer Foundation
- National Institute on Aging Information Center
- National Prostate Cancer Coalition (NPCC)
- PROCURE: Prostate cancer. The healing begins with awareness.
- Prostate Cancer Treatment Information
- A massive aggregation of media articles and data collated by patients for patients & Forum for patients and carers
- PCA3: Helping you decide when prostate biopsy is right

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# NGEP, a Prostate-Specific Plasma Membrane Protein that Promotes the Association of LNCaP Cells

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## Abstract

**NGEP** is a prostate-specific gene identified by analysis of expressed sequence tag databases. RNA analysis revealed two spliced forms of NGEP mRNA: a short form encoding a soluble protein (NGEP-S) and a long form encoding a polytopic membrane protein (NGEP-L). Transient expression of myc epitope-tagged NGEP-L showed that it was localized to the plasma membrane. We have now produced a specific antibody to the COOH terminus of NGEP-L and showed that it detects an ~100-kDa protein in extracts of normal prostate and prostate cancers that contain high levels of NGEP mRNA. The antibody detects a protein that is highly expressed on the apical and the lateral surfaces of normal prostate and prostate cancer cells by immunohistochemistry. The antibody does not detect a protein in the prostate cancer cell line LNCaP, which has very low NGEP mRNA levels. To study NGEP function, two stable LNCaP cell lines were prepared by transfection with NGEP-L and shown to contain similar amounts of NGEP-L protein as human prostate. Confocal immunofluorescence showed that NGEP-L is present on the plasma membrane of the transfected LNCaP cells and is highly concentrated at cell:cell contact regions. Furthermore, as the cell density increased, the cells formed large aggregates. A specific RNA interference that lowered NGEP-L levels prevented formation of cell aggregates. Our results suggest that NGEP-L has a role in promoting cell contact-dependent interactions of LNCaP prostate cancer cells and also that NGEP is a promising immunotherapy target for prostate cancer. [Cancer Res 2007;67(4):1594–601]

## Introduction

Adenocarcinoma of prostate is the second leading malignancy in men in United States. It was estimated that in the year 2006 in the United States, 234,460 men would be diagnosed with prostate cancer and 28,000 would die of the disease (1).<sup>3</sup> Despite recent advances in diagnosis and treatment, current therapies are unable to completely eliminate the androgen-independent prostate cancer cells that remain after androgen ablation (2–4). To develop improved treatments for prostate cancer, it is important to identify and characterize new molecular targets. Our laboratory has used a computer-based strategy of searching for expressed sequence tags (EST) that are expressed in prostate tissues to identify new genes

that are expressed in prostate cancer and not in essential normal tissues (5). One of the genes discovered by this approach is **NGEP**.

The **NGEP** gene, also now known as *TMEM16G*, is located on chromosome 2 at 2q37.3. There are two spliced forms of NGEP mRNA. The smaller transcript encodes a 179-amino acid cytoplasmic protein (NGEP-S) and the larger transcript encodes a 933-amino acid polytopic membrane protein (NGEP-L). RNA analysis has shown that NGEP is only detected in prostate samples [normal, benign prostate hyperplasia (BPH), and cancer], indicating it is a differentiation antigen made in normal prostate that continues to be expressed in cancers (1). In an initial attempt to localize the NGEP-L protein, we transfected 293T cells with a myc-tagged NGEP-L cDNA and showed that the protein was localized to the plasma membrane (6). The current study was undertaken to address the question of the location and possible function of NGEP-L in a prostate cancer cell line. Our study shows that in LNCaP cells expressing high levels of NGEP-L, the protein is concentrated at cell:cell contact regions where it seems to promote the association of cells into aggregates and that this aggregation is specifically prevented by RNA interference (RNAi), which dramatically lowers NGEP-L protein levels. This is the first study showing the presence of a prostate-specific protein in the cell:cell contact regions of prostate cancer cells and raises the possibility that NGEP-L may be responsible for cell contact-dependent interactions in the epithelial cells of the prostate.

## Materials and Methods

**Materials.** DMEM and LipofectAMINE were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). Fatty acid-free bovine serum albumin (BSA), Triton X-100, octyl glucoside, and protease inhibitors were from Sigma (St. Louis, MO). Tissues were obtained from Cooperative Human Tissue Network (Charlottesville, VA).

**Identification of NGEP-L related proteins in the human genome.** The sequences of NGEP-L related proteins were collected by a BLAST search of a nonredundant human protein database or a BLAT search of the human genome assembly using the NGEP-L protein sequence as a query. Multiple sequence alignment of NGEP-L and related proteins were prepared using T-Coffee (7) and manually adjusted and visualized using CHROMA (8).

**Generation of the Rab-Fc-NGEP-L (875–933) fusion protein.** The COOH terminus of the NGEP-L (residues 875–933) was expressed in transfected human kidney 293T cells as a fusion protein with a rabbit immunoglobulin (IgG) 1 Fc fragment. The expression vector for the Rab-Fc-NGEP-L (875–933) was produced by subcloning the NGEP-L fragment 875 to 933 in the pSec-Tag Rab-Fc vector using plaque-forming units (PFU)

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<sup>3</sup>National Center for Health Statistics, Division of Vital Statistics, Centers for Disease Control [Accessed 2006 January]. Available from: <http://www.cdc.gov/nchs/nvss.htm>.

Polymerase (Stratagene, La Jolla, CA). The PCR products were purified using QIAquick Purification kit (Qiagen, Valencia, CA), digested with *Sac*II and *Xba*I, and cloned in pSec Tag-Rab-Fc digested with *Sac*II and *Xba*I. The plasmids were sequenced in the regions that underwent genetic manipulations. The Rab-Fc-NGEP-L (875–933) plasmid was transfected in 293T cells using LipofectAMINE Plus (Invitrogen) and the manufacturer's protocol. Supernatants from the transfected cells were collected for 5 days beginning 48 h post-transfection and purified on a protein A column (Immunopure Plus protein A, Pierce, Rockford, IL). Protein concentrations were measured by a Coomassie protein assay reagent (Pierce) according to the manufacturer's protocol and checked on SDS-PAGE gels.

**Generation of NGEP-L (875–933) 6 $\times$ -His fusion protein.** Expression vectors for the NGEP-L (875–933) 6 $\times$ -His was constructed by subcloning the NGEP-L (875–933) sequences of human NGEP-L into the pET28a vector (Novagen, Madison, WI) between *Nde*I and *Xba*I sites by overlap extension PCR using *Pfu* polymerase. The vector is designed to introduce a COOH-terminal His<sub>6</sub> tag between the *Xba*I site and the stop codon for affinity purification of expressed proteins.

*Escherichia coli* strain BL21 ( $\lambda$ DE3; Novagen) was used as a host for protein expression. Five hundred milliliters of Luria broth supplemented with 50  $\mu$ g/mL kanamycin were inoculated with 1 mL of overnight culture grown at 37°C. Cells were grown at 37°C until their absorbance at 600 nm reached ~0.6, and the protein expression was then induced with 0.5 mmol/L isopropyl-1-thio- $\beta$ -galactopyranoside (Research Products, Mount Prospect, IL). After 4 h, cells were harvested by centrifugation at 5,000  $\times$  g and 4°C for 10 min. Cells were resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.4% (v/v) Triton X-100, 0.4% (w/v) sodium deoxycholate, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). After the suspension was sonicated, the inclusion body pellet was obtained by centrifugation at 50,000  $\times$  g for 15 min at 4°C. The pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.8% (v/v) Triton X-100, and 0.8% (w/v) sodium deoxycholate. After centrifugation at 50,000  $\times$  g for 15 min at 4°C, the pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl and 8 mol/L urea. Protein was purified using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. Purity of protein samples determined electrophoretically was >90%. Aliquots of purified proteins were stored at 20°C.

**Polyclonal antibody production and purification.** One hundred micrograms of Rab-Fc-NGEP-L (875–933) were injected into rabbits with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent immunizations. Sera were collected after the fourth and sixth immunizations and analyzed by ELISA against the *E. coli*-purified NGEP-L (875–933) 6 $\times$ -His. An unrelated protein POTE-His, also made in *E. coli*, was used as a control to estimate the nonspecific signal coming from the antisera. Further, to see the cross-reactivity of the antisera, a competition assay was done using Rab-Fc-NGEP-L (875–933). The antibody was further purified by using a Superdex 200 column from which the IgG fraction was collected and concentrated.

**Western blotting analysis.** Human prostate tissue lysate was prepared by pulverization of the frozen prostate samples in lysis buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8), 0.8% Triton X-100, 0.2% NP40, 1% sodium deoxycholate, 1 mmol/L PMSF, 5  $\mu$ g/mL aprotinin, 5  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL pepstatin]. Tissue and cell debris were removed by centrifugation of the lysate at 14,000 rpm for 10 min. Protein concentration was determined by Coomassie protein assay reagent according to the manufacturer's protocol.

Fifty micrograms of protein extract from different prostate tissues were separated on a 4% to 20% Tris-glycine gel (Invitrogen) and transferred to a 0.2- $\mu$ m immunoblot polyvinylidene difluoride membrane (Invitrogen) in transfer buffer [25 mmol/L Tris/192 mmol/L glycine/20% (v/v) methanol (pH 8.3)] at 4°C for 2 h at 50 V. Filters were probed with 1:1,000 rabbit antiserum or preimmune serum. Primary antibodies were detected with the donkey antirabbit secondary antibody conjugated with horseradish peroxidase (HRP; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and their respective signals were detected by using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences).

**Generation of stable cell lines and RNAi.** LNCaP cells were grown in RPMI 1640 containing 10% FBS, 1 mmol/L pyruvate, 2 mmol/L glutamine, and 100  $\mu$ g/mL penicillin and 100  $\mu$ g/mL streptomycin in a 5% CO<sub>2</sub>/95% air humidified incubator at 37°C. For stable cell line generation, the cells at 80% confluence in six-well plates were incubated in serum-free and antibiotic-free RPMI 1640 containing 4  $\mu$ L LipofectAMINE and 6  $\mu$ L Plus reagent with 6  $\mu$ g DNA. Cells were incubated for 4 h at 37°C, after which complete medium was added to bring the final concentration to 10% FBS. Twenty-four hours after the start of the transfection, cells were passaged and split into 10 different plates at different dilutions. At 48 h, the medium was replaced with complete medium containing 750  $\mu$ g/mL G418 for the selection of the stable clones. Multiple G418 colonies were picked and the clones expressing NGEP-L were selected using Western blot analysis with the polyclonal antibody against NGEP-L.

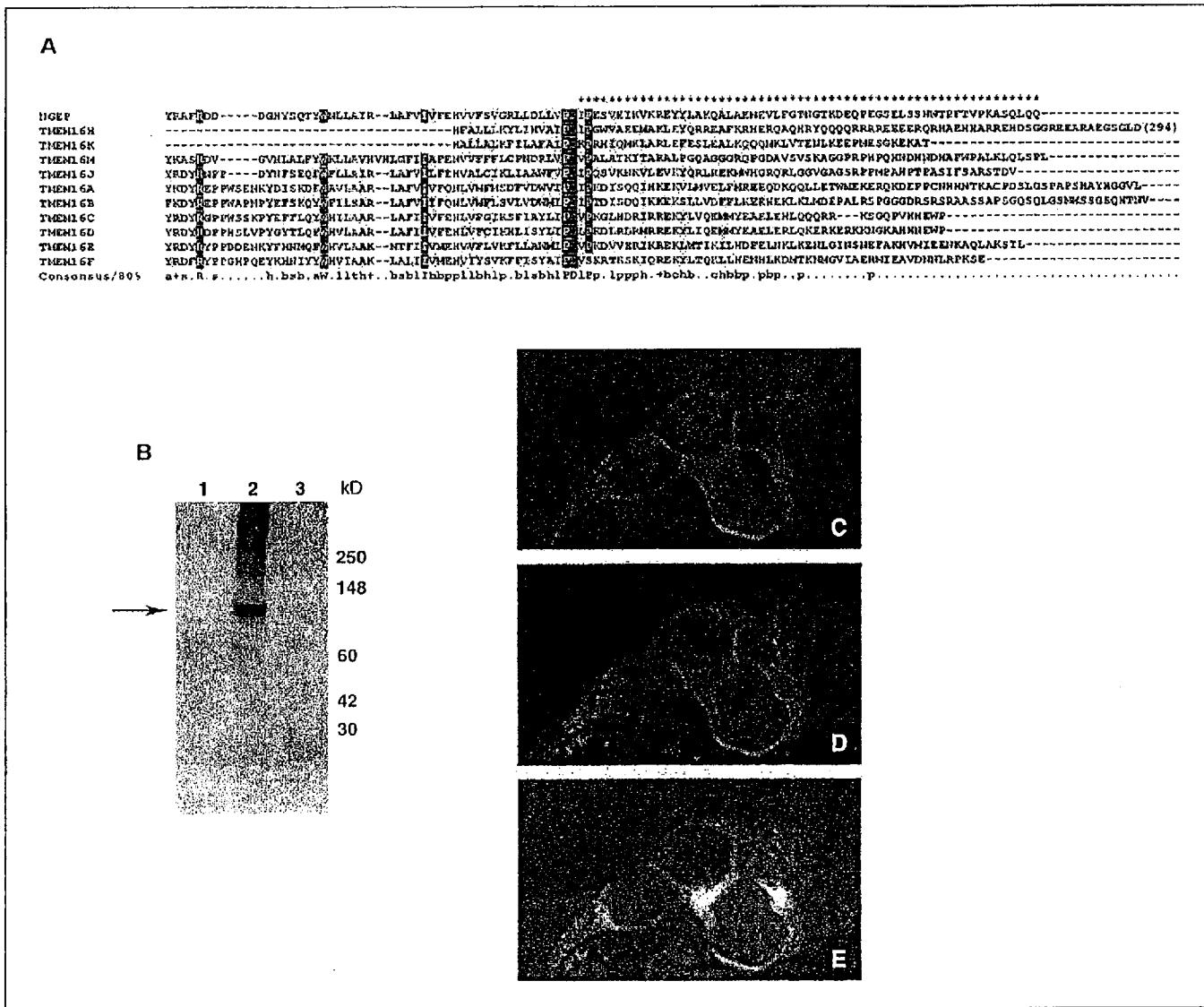
For NGEP-L RNAi experiments, LNCaP-CL-2 cells were transfected with 200 pmol NGEP-L-specific (D-023184-01) or control small interfering RNAs (siRNA; GL2-Luc; Dharmacon, Chicago, IL) using LipofectAMINE 2000 transfection reagent (Invitrogen) 48 h after plating of the LNCaP-CL-2 cells. Western analysis was done on the cells transfected with the siRNA and the extent of the inhibition of NGEP-L expression was studied. Each transfection was repeated twice in duplicate.

**Immunocytochemistry.** Cells were plated onto a LabTek chamber slide and grown for 2 days. After 48 h, cells were fixed for 20 min in 4% formaldehyde, treated for 10 min with 0.1% Triton X-100 in PBS, blocked for 30 min with 10% normal goat globulin in PBS, and then incubated at room temperature for 1 h with 1:100 diluted polyclonal antibody for NGEP-L. Subsequently, the cells were incubated at room temperature for 1 h with tetramethyl rhodamine-conjugated secondary antibodies (Invitrogen) at the concentration of 2  $\mu$ g/mL and then mounted in antifade solution with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Labeled cells were analyzed by laser confocal microscopy using a  $\times$ 63 oil immersion objective lens. For labeling of the endoplasmic reticulum (ER) and *cis*-golgi, 1:100 anti-protein disulfide isomerase (PDI) monoclonal antibodies (mAb; Abcam, Cambridge, MA) and 1  $\mu$ g/mL anti-human golgin-97, mouse monoclonal CDF4 (Invitrogen) were used, respectively, as the primary antibody. The secondary antibody used for the detection of ER and golgi was Alexa 488-labeled goat anti-mouse antibody (Invitrogen).

**Immunohistochemistry.** Paraffin-embedded prostate tissues were cut into 5-micron sections and mounted onto polylysine slides (Histoserv, Germantown, MD). The sections were deparaffinized in xylene followed by graded ethanol hydration into water. The sections were treated with antigen-retrieval solution (DAKO Genpoint, Carpinteria, CA) at 85°C for 20 min. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxidase for 10 min followed by washing in water twice for 5 min. Nonspecific labeling was blocked with 5% BSA in TBS for 20 min. After washing the sections in TBST for 5 min, tissues were incubated with polyclonal antibody against NGEP-L (dilution 1:1,500) in blocking buffer overnight at room temperature. After incubation, the sections were washed twice with TBST for 5 min. Sections were then treated with biotinylated goat anti-rabbit IgG (dilution 1:500; DAKO Genpoint) for 20 min at room temperature, washed once with TBST, and incubated with streptavidin HRP (dilution 1:400) for 30 min. The sections were washed once with TBST for 5 min and the secondary antibody was detected with 3',3'-diaminobenzidine peroxidase substrate (Sigma). Slides were counterstained with hematoxylin (Histoserv, Germantown, MD), dehydrated in graded ethanol and cleared in xylene, and mounted using Permount (Fisher Scientific, Pittsburgh, PA).

## Results

To study the cellular location and function of NGEP-L protein, we generated a specific polyclonal antibody to NGEP-L. Because NGEP-L is a member of the TMEM16 protein family, we used the data of Galindo and Vacquier (9) and two additional members identified in this study to choose a region of NGEP-L that is different from other family members. Our sequence comparison of the TMEM16 family members showed that among the paralogs, the



**Figure 1.** Generation of antibodies against NGEP. *A*, multiple sequence alignment of COOH termini of 11 human NGEP-L related proteins (TMEM16 family). Amino acid residues encoded by exons 22 to 25 of NGEP-L (amino acids 829–933) and its homologous regions in NGEP-L related proteins are shown. TMEM16H and TMEM16K lack the exon equivalent to NGEP-L exon 22. \*, peptide sequence used for antibody production. The unaligned residues of TMEM16H are omitted (parenthesis). Markings in the consensus line are as follows: capital letters, amino acid residue identical in 80% or more sequences; *i*, aliphatic (ILV); +, positive (HKR); *t*, tiny (AGS); *a*, aromatic (FWY); *c*, charged (DEHKR); *s*, small (ACDGNPSTV); *p*, polar (CDEHKNQRST); *b*, big (EFHIKLNMQRWY); *h*, hydrophobic (ACFGHILMTVWY). The GenBank gi number and amino acids shown here are as follows: TMEM16A, 40354210:838–960; TMEM16B, 9966845:876–999; TMEM16C, 13899227:887–981; TMEM16D, 30520318:823–920; TMEM16E, 47106048:806–913; TMEM16F, 70778895:808–910; NGEP-L (TMEM16G), 49533623:829–933; TMEM16H, 55741655:851–1232; TMEM16J, 59858803:680–782; TMEM16K, 27545336:332–392; and TMEM16M, 51459226:477–584. *B*, Western blot analysis of NGEP-L-transfected cell extracts with an NGEP-L antibody. The expected 100-kDa protein is detected in extracts transfected with pcDNA3.1-NGEP-L myc (lane 2). A cell extract from vector only-transfected cells or pcDNA3.1-POTE21-transfected cell produced no signal (lanes 1 and 3, respectively). Forty micrograms of protein lysate were loaded onto a 4% to 20% gel and Western blot analysis was done using NGEP polyclonal antibody at a dilution of 1:1,000. HRP-labeled donkey antirabbit secondary antibody at a dilution of 1:2,000 was used for detection. *C* to *E*, 293T cells were transiently transfected with a plasmid encoding EGFP-NGEP. After 24 h, the cells were stained with anti-NGEP antibodies. The fluorescence of EGFP-NGEP in the 293T cells expressing the fusion protein (*C*; green) completely colocalized with the anti-NGEP (*D*; red) as shown in (*E*; yellow). Nuclei were stained with DAPI (blue).

COOH termini have high sequence diversity (Fig. 1A). Based on this information, we chose residues 875 to 933 to generate NGEP-L antibodies.

**Preparation of Rab-Fc-NGEP-L (875–933) protein and generation of the polyclonal antibodies.** Rabbits were immunized with a Rab-Fc-NGEP-L (875–933) fusion protein composed of the Fc fragment of rabbit IgG1 and the COOH-terminal amino acids (875–933) of NGEP-L as described in Materials and Methods. Blood

was collected from the rabbits after the fourth and sixth immunizations and the antibody titer and specificity were determined by ELISA on plates coated with NGEP-L (785–933) 6×-His protein made in *E. coli* (data not shown). The antibody was purified on a Superdex 200 column to remove albumin and other proteins and the IgG fraction was collected and concentrated.

The minimum amount of recombinant NGEP-L (785–933) that could be detected on a Western blot by the purified polyclonal

antibody was determined using different dilutions of polyclonal antibody and different concentrations of NGEP-L (785–933) 6×His. Using Western blot analysis, the NGEP-L antisera at a dilution of 1:1,000 could detect 1 ng of recombinant NGEP-L (785–933; data not shown). To determine if the polyclonal NGEP-L antibody could detect NGEP-L protein in mammalian cells, 293T cells that do not express NGEP-L were transfected with a pcDNA3.1/NGEP-L-myc plasmid. Total lysates were subjected to SDS-PAGE. As shown in Fig. 1B, the polyclonal antibody specifically detected a strong band of ~100 kDa in 293T cells transfected with pcDNA3.1 NGEP-L-myc. It also detected higher molecular bands very likely representing aggregated protein (Fig. 1B, lane 2). (Aggregation of polytopic proteins is often observed when these proteins are produced at high levels by transfection.) No signal was detected in the untransfected cells (Fig. 1B, lane 1) or in 293T cells expressing a cDNA encoding a control protein POTE21 (Fig. 1B, lane 3; ref. 10). These data establish that the polyclonal antibody against NGEP is specific and can detect NGEP-L expressed in mammalian cells.

We showed previously that NGEP-L tagged with myc localizes in the plasma membrane of transfected 293T cells (6). To determine if the polyclonal antibody detects the same protein, we chose to immunologically colocalize transfected NGEP-L by transfecting 293T cells with enhanced green fluorescent protein (EGFP)-NGEP-L and determining if the EGFP signal (green) colocalizes with one produced by an antibody to NGEP-L detected with a tetramethyl rhodamine goat anti-rabbit antibody (red). The images in Fig. 1C–E show that the two signals overlap, indicating the NGEP-L antisera detects NGEP-L in transfected cells (Fig. 1E). No signal was obtained from the untransfected 293T cells or when prebleed sera was used for the immunofluorescence on NGEP-L-transfected cells

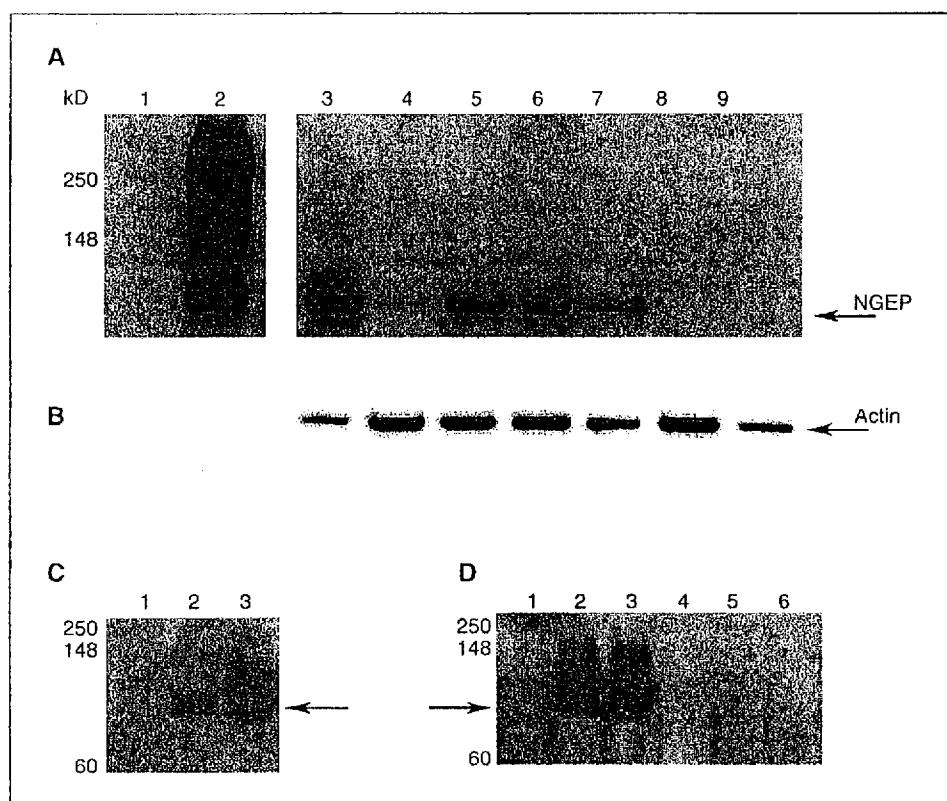
(data not shown), showing the antibody is specific to NGEP-L and can detect NGEP-L in formalin fixed cells.

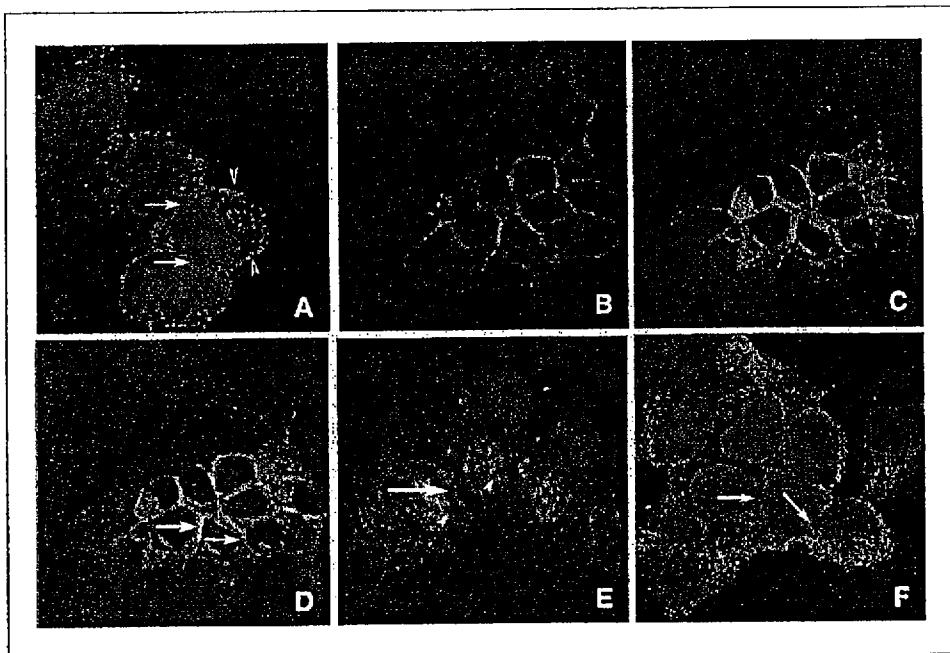
**Expression of NGEP in prostate tissue.** Because RNA studies have shown that *NGEP-L* is a prostate-specific gene (6), we used the antisera to test for the presence of NGEP-L in protein extracts of normal prostate and prostate cancer and a few normal tissues. Fifty micrograms each of tissue lysates were resolved on a 10% SDS gel, blotted, and then reacted with the polyclonal NGEP-L antibody. As shown in Fig. 2A, a band of ~100 kDa was detected in protein extracts of 293T cells transfected with pcDNA3.1 NGEP-L-myc (Fig. 2A, lane 2) as well as bands in the high molecular weight regions due to aggregated protein. A specific band at the expected  $M_r$  of ~100 kDa was detected in tissue extracts from normal prostate (Fig. 2A, lane 3), BPH (Fig. 2A, lanes 4 and 5), and prostate cancers (Fig. 2A, lanes 6 and 7). The expression of NGEP-L in one of the BPH samples was low (Fig. 2A, lane 4). No signal was detected in liver and brain tissue lysates. To evaluate the quality of the lysates, we did a Western blot with a β-actin antibody as shown in Fig. 2B; a similar amount of actin was detected in all the tissue lysates. We conclude the antisera can specifically detect NGEP-L in prostate tissue lysates.

**Generation of NGEP-L-expressing prostate cell line.** Previously, we found that NGEP mRNA is present at extremely low levels in LNCaP cells using reverse transcription-PCR. This level is much less than the levels present in tissue and tumor samples from patients, suggesting NGEP-L expression was lost when the LNCaP cell line developed. As expected, attempts to detect NGEP-L protein in LNCaP cells by Western blot were unsuccessful (data not shown).

To study the location and possible function of NGEP-L in prostate cancer cells, we established two stable LNCaP cell lines expressing NGEP-L by transfecting NGEP-L into these cells using a

**Figure 2.** Western analysis of NGEP-L in prostate tissue and prostate cancer cell lines. *A*, Western blot analysis of NGEP-L expression: 50 μg of lysate from 293T nontransfected 293T cells (lane 1), 30 μg of cell lysate from 293T transfected with cDNA encoding NGEP-L (lane 2), and 50 μg of tissue lysate from normal prostate (lane 3), BPH (lanes 4 and 5), prostate cancer (lanes 6 and 7), brain (lane 8), and liver (lane 9) were analyzed by Western blotting using rabbit anti-NGEP-L antibody (1:1,000). *B*, the blot was probed with anti-β-actin (1:250; Abcam) as a loading control. *C*, Western analysis of NGEP-L expression in the stable cell lines. The samples (40 μg) loaded are the following: 1, LNCaP-V; 2, LNCaP-CL-1; and 3, LNCaP-CL-2. A protein band ~100 kDa was observed for NGEP-L-transfected cells. *D*, subcellular fractionation was done using differential centrifugation in LNCaP-CL-2 and LNCaP-V. The samples (40 μg cells extracts) loaded are the following: 1, LNCaP-CL-2 cytosol fraction; 2, LNCaP-CL-2 nuclear fraction; 3, LNCaP-CL-2 membrane fraction; 4, LNCaP-V cytosol fraction; 5, LNCaP-V nuclear fraction; and 6, LNCaP-V membrane fraction. NGEP-L is present in the crude membrane and nuclear fractions but not in the cytosolic fraction.





**Figure 3.** NGEP-L is present in the cell:cell contact regions in the LNCaP-CL-2 cells. *A*, immunofluorescence of NGEP-L was done on LNCaP-CL-2 using the rabbit NGEP-L antibody and the signal was detected using tetramethylrhodamine goat antirabbit. Arrows, NGEP-L (red) was predominantly localized in the cell:cell contact regions. There was also presence of NGEP-L in the plasma membrane (arrowheads) and also intracellular organelles. *B* to *D*, the LNCaP-CL-2 cells were stained with tetramethylrhodamine B isothiocyanate-labeled phalloidin (*B*; red) and the immunofluorescence of NGEP-L was done using rabbit NGEP-L antibody followed by Alexa 488-labeled goat antirabbit antibody (*C*; green). Phalloidin labels the F-actin beneath the plasma membrane and NGEP-L is present in the membrane of the cells. Arrows, NGEP-L is predominant in the cell:cell contact regions (*D*; yellow). *E*, LNCaP-CL-2 cells were stained with golgin-97 antibody, a *cis*-golgi protein (green), and costained with anti-NGEP-L antibody (red). Arrowhead, colocalization (yellow) of golgin-97 and NGEP; arrow, localization of NGEP in the cell-cell contact regions. *F*, LNCaP-CL-2 cells were stained with PDI antibody, an ER protein (green), and costained with anti-NGEP antibody (red). The nuclei were stained with DAPI.

pCDNA3.1-based vector as described in Materials and Methods. As shown in Fig. 2*C*, NGEP-L expression in Cl-2 (Fig. 2*C*, *lane 3*) is higher than expression in Cl-1 (Fig. 2*C*, *lane 2*). In addition, there is no detectable expression of NGEP-L in a vector-transfected LNCaP-V cell (Fig. 2*C*, *lane 1*). These cell lines were used for further experiments.

**NGEP-L is a plasma membrane protein.** To verify that NGEP-L was tightly associated with the membrane fraction of cells, LNCaP-CL-2 cells were disrupted by homogenization and subjected to low- and high-speed centrifugation, and the samples were analyzed by Western blots. As shown in Fig. 2*D*, NGEP-L was detected in both the low (Fig. 2*D*, *lane 2*) speed ( $1,000 \times g$ ) and the high (Fig. 2*D*, *lane 3*) speed membrane fractions ( $100,000 \times g$ ) but not in the soluble fraction of the cells (Fig. 2*D*, *lane 1*). In both membrane fractions (Fig. 2*D*, *lanes 2* and *3*), we observed a major band of the expected size ( $\sim 100$  kDa) for NGEP-L and a higher molecular weight band (probably an aggregate), which was not present in any of the fractions of the vector-transfected cells (Fig. 2*D*, *lanes 4*–*6*).

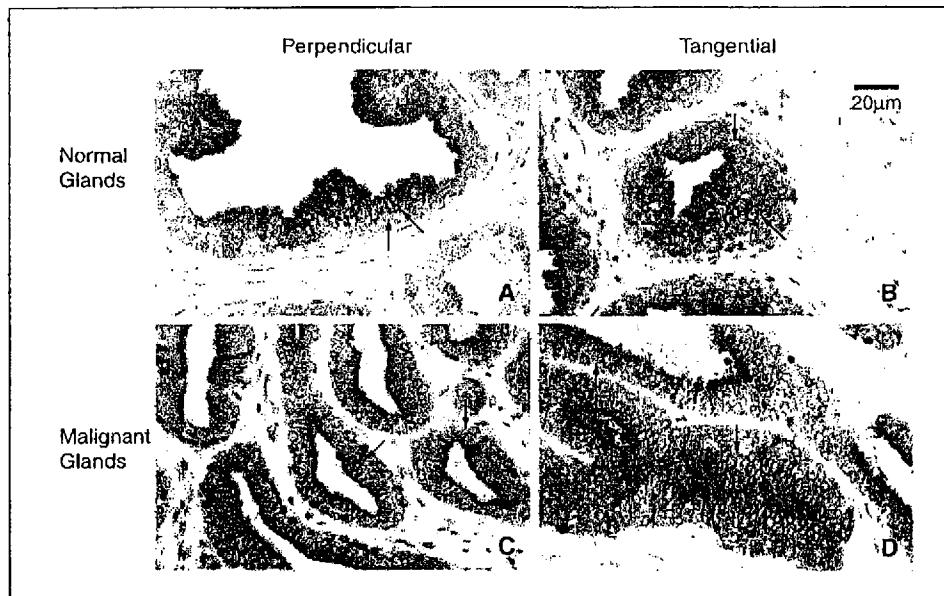
We next did immunofluorescence studies with LNCaP-CL-2 cells. As shown in Fig. 3*A*, there is strong red fluorescence signal (tetramethylrhodamine) in the cell:cell contact regions and a weaker signal in the regions of the plasma membrane where the cells are not touching other cells. In addition, there is some punctate staining in the interior portion of the cell. To confirm the plasma membrane location of NGEP-L, we double stained cells using Alexa 488 (green) to stain NGEP-L and tetramethylrhodamine B isothiocyanate-labeled phalloidin to stain the F-actin network that lies very close to the plasma membrane. As shown in

Fig. 3*B*, phalloidin stains the actin present adjacent to the plasma membrane region. Figure 3*C* shows the green NGEP-L signal that is very strong in cell:cell contact regions but also present inside the cell. Figure 3*D* shows that there is very good colocalization of NGEP-L and actin in the region of the cell membrane as would be expected at this level of resolution. The bright yellow signal at cell junction regions is consistent with an increase in NGEP-L in that region. There was no NGEP-L signal detected when we analyzed LNCaP-V cells or in LNCaP-CL-2 cells using prebleed sera establishing the specificity of the signal observed (data not shown).

To determine the location of NGEP-L protein within the cell, we did double labeling experiments with a *cis*-golgi marker (golgin-97) and with an ER marker (PDI) in LNCaP-CL-2 cell. As shown in Fig. 3*E*, there is colocalization of some of the intracellular NGEP-L signal with the *cis*-golgi marker but not with the ER marker (see Fig. 3*F*). It is likely that this signal represents NGEP-L being processed in the Golgi compartment before delivery to the cell membrane.

To determine the location of NGEP in normal prostate and prostate cancer, we did immunohistochemistry using four different formalin-fixed, paraffin-mounted prostate cancer specimens obtained from radical prostatectomy. Figure 4 shows the staining of the NGEP in both normal and malignant glands from one of the prostate cancer specimens. In the normal glands, NGEP is present at the apical and the lateral intercellular region between acinar epithelial cells, when sectioned perpendicular to the basement membrane (Fig. 4*A*). Figure 4*B* shows the same expression pattern, when sectioned tangential to the basement membrane. Figure 4*C* and *D* shows the perpendicular and the tangential sections from

**Figure 4.** Location of NGEP-L in prostate. Slides were prepared from formalin fixed, paraffin-embedded human prostate cancer samples. NGEP-L was detected as described in Materials and Methods using a polyclonal antibody to NGEP-L at a dilution of 1:1,500. Arrows, NGEP-L signal on the lateral surface of the cells. *A* and *B*, normal prostate. *C* and *D*, prostate cancer. The signal is visible in both perpendicular and tangential sections of normal and malignant prostate glandular epithelia with respect to the basement membrane on which the epithelial cells sit. Perpendicular sections (*A* and *C*). Tangential sections (*B* and *D*).



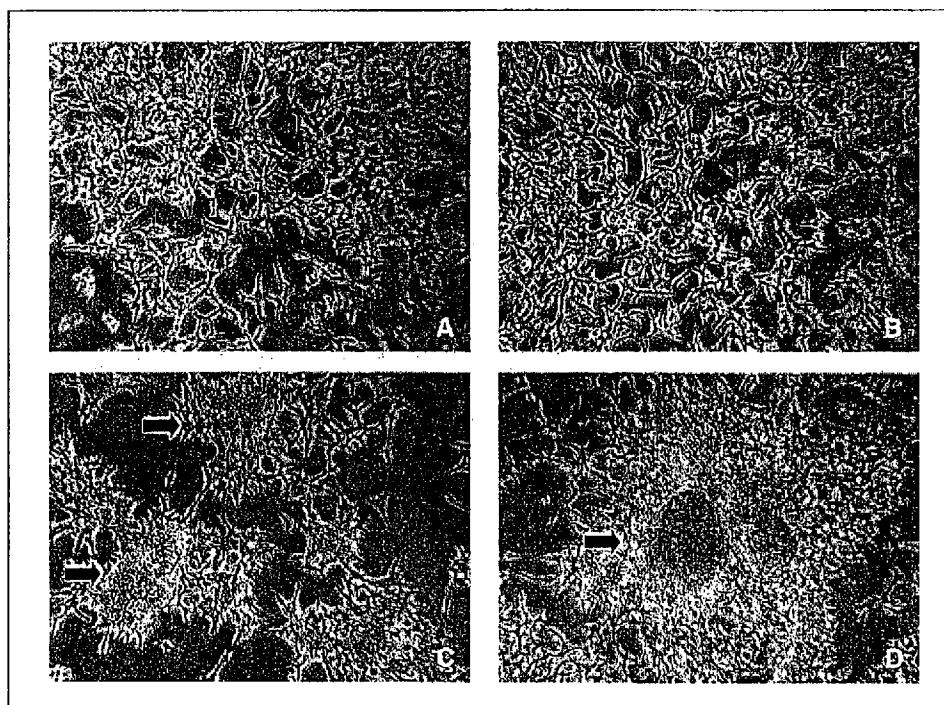
malignant prostate adenocarcinoma glands, respectively. In the malignant prostate glands, NGEP was expressed on the lateral and the apical surface of the epithelial cells, similar to the distribution in normal prostate glands.

**Effect of NGEP-L on cell morphology.** To examine if expression of NGEP-L in the cell:cell contact regions can alter the behavior or shape of the LNCaP cells, we plated  $4.5 \times 10^4$  cells from each of the two clones in a six-well plate and observed the cells for several days. Figure 5 shows phase-contrast micrographs of living cells taken on day 7. Two striking differences were observed when compared with untransfected LNCaP cells (Fig. 5*A*) or

LNCaP-V cells (Fig. 5*B*). Cells expressing NGEP-L protein formed large aggregates and the cells not in aggregates were rounder and less spread out than control cells (Fig. 5*C* and *D*). This behavior was not due to clonal variation because we made the same observation in two different cell lines.

**RNAi-mediated inhibition of NGEP-L expression.** To determine if the aggregation and morphologic changes of the NGEP-L-expressing cells were due to expression of NGEP-L, we carried out siRNA experiments to lower the NGEP-L levels. We reasoned that if the change in the morphology of the cells is due to NGEP expression, then down-regulation of NGEP-L would revert the cell

**Figure 5.** Expression of NGEP-L in the LNCaP cells alters the morphology of the cells. Phase-contrast photographs of growing LNCaP cells. *A*, parental LNCaP cells. *B*, LNCaP-V cells. *C*, LNCaP-CL-1. *D*, LNCaP-CL-2. NGEP expression shows a circular morphology, more cell:cell adhesion morphology compared with the control cells. Arrows, aggregation of the cells due to NGEP-L.



shape to that of the parental LNCaP cells and eliminate the aggregates. Four siRNA oligonucleotide sets were tested for their ability to lower NGEP-L levels measured on a Western blot before deciding on the best construct for the efficient knockdown. siRNA (D-023184-01) specific to NGEP-L was chosen and was used for the studies shown here. To show down-regulation of the NGEP-L protein level in the LNCaP CL-2 cells, Western blot analysis was carried out. As shown in Fig. 6A, NGEP-L siRNA repressed the expression of NGEP-L protein in the LNCaP-CL-2 cells (Fig. 6A, lanes 1–3), but the control siRNA did not (Fig. 6A, lane 4). The maximum reduction in protein levels was observed 4 days after transfection.

To determine if the down-regulation of NGEP-L protein has any effect on the aggregation or morphology of the LNCaP-CL-2 cells, we monitored the cells after transfection with the siRNAs. Figure 6B to G shows the phase-contrast micrographs of the LNCaP-CL-2 cells transfected with NGEP-L-specific siRNA, control siRNA, and the nontransfected cells at days 4 and 6 after transfection. The LNCaP-CL-2 cells transfected with siRNA-NGEP-L show a morphology that is similar to that of the parental untransfected LNCaP cells (Fig. 6B and E and compare it with Fig. 5A and B). The cells are more elongated compared with the LNCaP cells expressing NGEP-L. The LNCaP-CL-2 cells (Fig. 6D and G) and the LNCaP-CL-2 cells transfected with nonspecific siRNA (Fig. 6C and F) have a similar morphology. They form clumps of cells as shown in Fig. 6C, D, F, and G, arrows.

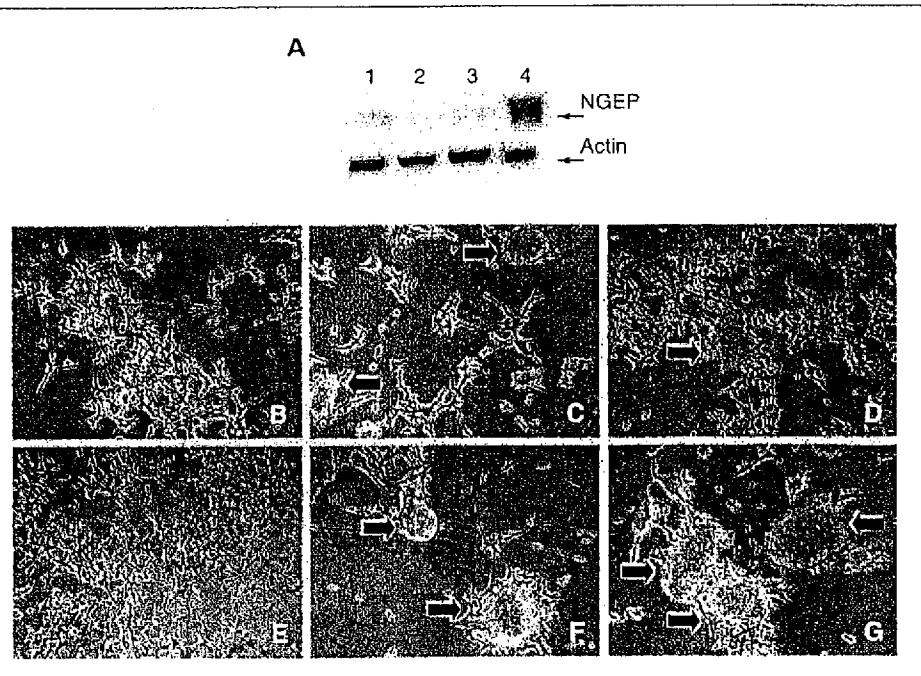
To determine if the NGEP-L interaction responsible for the cell:cell adhesion requires calcium, we did  $\text{Ca}^{2+}$  chelation with EGTA. We reasoned if the cell:cell adhesion due to NGEP-L requires calcium, depletion of the extracellular calcium will cause the aggregates to come apart. We treated the LNCaP-CL-2 cells with 2 mmol/L EGTA for 1 h and monitored the cells. We found that EGTA treatment caused the LNCaP-CL-2 cells to detach from the dish. However, the few cells that remained on the plate were still in clumps (data not shown).

## Discussion

The NGEP gene was originally identified by analyzing the EST database and searching for genes expressed in prostate and prostate cancer and not other tissues. Proteins encoded by such genes could be useful therapeutic targets for cancer therapy. An analysis of NGEP mRNA showed that there are two spliced variants (6). One of these encodes a short cytoplasmic protein (NGEP-S) and the other encodes a long polytopic membrane protein (NGEP-L), which is a member of the TMEM16 family of proteins (9, 11). The antibody used in the current study was generated against the COOH terminus of NGEP-L because that region is the most diverse among the family members. The antibody specifically detected a ~100-kDa protein in samples of normal prostate, prostate cancer, and BPH but not in other tissues examined, such as liver and brain. This finding indicates that the antibody is specific for NGEP-L. We then examined two prostate cancer cell lines, PC3 and LNCaP, for NGEP-L protein expression and it was not detected in either. This result was not surprising because NGEP mRNA is not detected in PC3 cells and is expressed at a very low level in LNCaP cells (6). We then used LNCaP cells to evaluate NGEP-L function because it is more differentiated than the PC3 line as evidenced by its production of the prostate-specific antigen and its response to androgen (12).

Two cell lines producing NGEP-L were established by transfecting a full-length NGEP-L cDNA into LNCaP cells. Western blots showed that these cell lines produced NGEP-L protein with the expected molecular weight and the levels were comparable with NGEP-L protein levels in prostate cancer specimens (Fig. 2C). Examination of the location of NGEP-L by confocal microscopy using the antibody to NGEP-L showed that NGEP-L was located in the plasma membrane and that its concentration was increased at cell contact regions. Furthermore, as the cells grew to high density, large aggregates of cells formed, suggesting that NGEP-L has an important role in promoting cell:cell interactions. These large

**Figure 6.** NGEP-L siRNA mediates gene silencing in LNCaP-CL-2 cells. *A*, Western blot analysis of the NGEP-L protein expression was done after 2, 4, and 6 d of transfection with RNA duplexes. A control siRNA (GL2-Luc) was used in parallel to test potential nonspecific effects of the short RNA duplexes. The unaffected actin expression was used as internal standard of protein concentration in each lane. Twenty micrograms of cell lysates after 2 d of siRNA-NGEP transfection (lane 1); 4 d of siRNA-NGEP transfection (lane 2); 6 d of siRNA-NGEP transfection (lane 3); and 6 d of siRNA-GL2-Luc transfection (lane 4) were loaded onto a gel and Western blot analysis was done using rabbit NGEP antibody (1:1,000). *B* to *G*, NGEP-L knockdown induces alteration in the morphology of the LNCaP-CL-2 cells. Phase-contrast photographs of growing LNCaP-CL-2 cells during NGEP gene silencing. LNCaP-CL-2 transfected with siRNA-NGEP at day 4 (*B*) and day 6 (*E*). LNCaP-CL-2 transfected with control siRNA (GL2-Luc) at day 4 (*C*) and day 6 (*F*). LNCaP-CL-2 cells at day 4 (*D*) and day 6 (*G*). Arrows, clump of cells present in the LNCaP-CL-2 and in the LNCaP-CL-2 cells transfected with nonspecific siRNA. The clumps disappeared in cells transfected with siRNA specific to NGEP-L.



aggregates were not observed in cells transfected with an empty vector or in nontransfected LNCaP cells. To confirm that aggregation was due to presence of NGEP-L protein, we showed that a RNAi for NGEP-L prevented the formation of large cellular aggregates.

We also examined the location of NGEP-L in both normal prostate and prostate cancers tissues using formalin fixed tissues obtained from prostatectomy samples. Figure 4 shows one example; the other three showed very similar results. In both normal and cancers, NGEP-L was localized on the apical and the lateral surfaces of the epithelial cells of prostate. This location is consistent with the finding with transfected LNCaP cells where accumulation of NGEP at cell contact regions was observed. The similar cell:cell contact location of NGEP-L in the LNCaP cells and in the prostate tissue suggests that NGEP-L may have an important role in the cell:cell interactions.

Connexins are gap junction proteins ubiquitously present in epithelial cells, including prostate, and have an important role in intercellular communication allowing the transfer of small molecules between cells. In many epithelial cancers, including prostate cancer, the levels of connexin are low or even absent (13–15). Connexin levels are low in LNCaP cells (16, 17) and overexpression of connexins has been shown to cause aggregation of LNCaP cells similar to that observed with NGEP-L.

transfected LNCaP cells (16). However, unlike NGEP-L, connexins are not differentiation antigens with a specific role in the prostate. It is possible that NGEP-L, perhaps cooperating with connexins, has an important role in the formation of the prostate gland. We are currently making NGEP knockout mice to examine this hypothesis.

NGEP-L is a polytopic protein but its precise orientation of the protein in the membrane has not been established. NGEP-S, which shares a common NH<sub>2</sub> terminus with NGEP-L, is a cytosolic protein and it is likely that the NH<sub>2</sub> terminus of NGEP-L is also inside the cell (6). The COOH terminus of NGEP-L is probably also inside the cell because the antibodies described in this article that react with the COOH terminus only detect NGEP in permeabilized cells. We are currently producing mAbs to other regions of NGEP to learn more about its function and its orientation in the plasma membrane. mAbs to an extracellular portion of NGEP could be useful in the immunotherapy of prostate cancer.

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NGF-L, a prostate specific plasma membrane protein, is a new target for prostate cancer immunotherapy.

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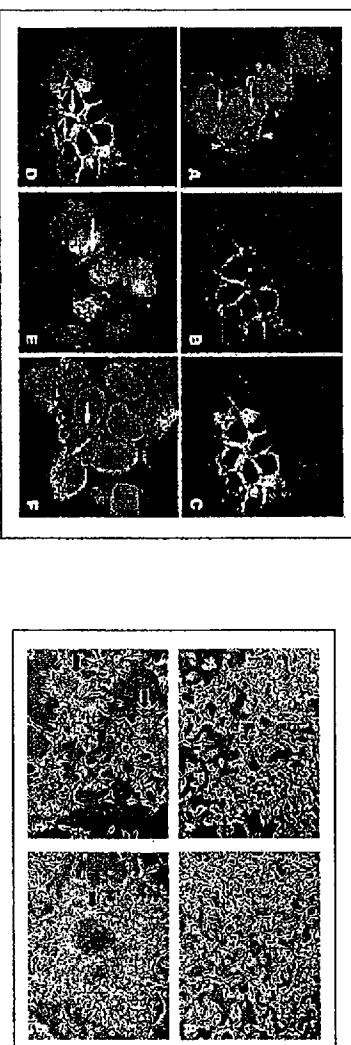
## Abstract

The expression of the NGEP<sub>L</sub> (MWB10) gene has only been detected in normal prostate and prostate cancer RNA analysis revealed 2 splice variants of NGEP<sub>L</sub>. A short form (NGEP<sub>S</sub>) encodes a 20 kDa cytosolic protein and a long form (NGEP<sub>L</sub>) encodes a 35 kDa polypeptide membrane protein. To study the location and the function of NGEP<sub>L</sub>, we have generated a panel of monoclonal and polyclonal antibodies to the different regions of the NGEP<sub>L</sub>. The antibodies detect a 35 kDa protein in extracts of normal prostate and prostate cancers that contain high levels of NGEP mRNA by immunodot blotting or the prostate tissues using antibodies against NGEP<sub>L</sub> shows that NGEP<sub>L</sub> is highly expressed on the apical surface of normal prostate and prostate cancer cells. Lateral expression suggests that NGEP<sub>L</sub> may have a role in prostate cell interactions or adhesion. To study the function of NGEP<sub>L</sub> two stable LUC<sub>2</sub> cell lines expressing NGEP<sub>L</sub> at levels similar to those found in prostate tissues were generated. Confocal immune-fluorescence studies of these cell lines present that NGEP<sub>L</sub> is present in the plasma membrane and is highly concentrated at the cell-cell contact regions. Furthermore, as the cells express NGEP<sub>L</sub> increase in density, the cells form aggregates. A specific RNA that covered NGEP<sub>L</sub> levels prevented formation of the cell aggregates. Our results suggest that NGEP<sub>L</sub> has a role in promoting cell-cell contact dependent interactions of prostate cells. Also, because of its high expression in prostate cancer, its specific expression in prostate and its presence on the cell surface, NGEP<sub>L</sub> is a promising target for the antibody-based therapies of prostate cancer.

ISSUE-SPECIFIC EXPRESSION OF NGEF

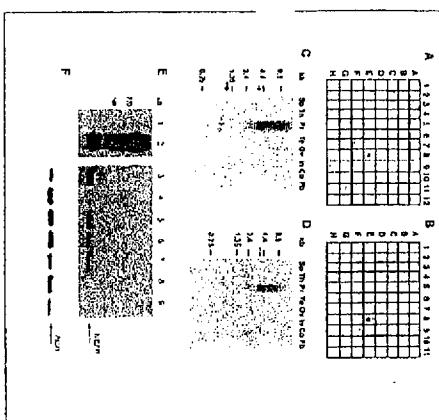
NGEP is localized in cell-cell contact regions

#### Expression of NGEF-Z causes aggregation



**Immunochemical analysis of NGFL-L with Mab 19C1.**

A. Immunoblotting lanes 1–4 show the protein profile in the cell culture supernatants of the indicated cell types. B. The same cell types were analyzed by electron microscopy. C. The same cell types were analyzed by immunofluorescence. D. The same cell types were analyzed by confocal microscopy. E. LNCaP-NCBP cells were stained with antibodies against p53, and co-stained with anti-p53 antibody (red) and anti-NCBP antibody (green). A. Arrowheads indicate bands of p53 and NCBP; arrow, localization of NCBP in the cell culture supernatants. F. LNCaP-CL-2 cells were stained with PGP antibody, an ER protein (green), and co-stained with anti-NCBP antibody (red).



**Fig. 1.** Tissue-specific expression of NGEP mRNA and protein.

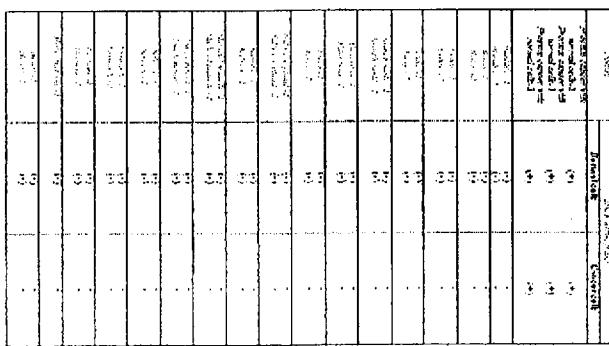


Fig. 3. Location of NGEP-L in prostate



**Fig. 5.** NGEP-1 siRNA mediated gene silencing in LNCaP-NGEP cells reverses the

epitope. A standard IgG-binding test was performed using purified IgG from patients with known IgG antibodies to the hepatitis C virus (HCV) envelope protein (E2). Positive sera were obtained from patients with chronic hepatitis C and negative sera were obtained from healthy donors. HCV antibody was detected by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Murex Biotech Ltd., U.K.). Positive sera were used at a dilution of 1:100 and negative sera at 1:1000. The IgG-binding test was performed in triplicate and each sample was tested in duplicate. The results were expressed as a mean value of IgG-binding activity. The positive control was a serum with a known titre of 1:1000. The negative control was a serum with a known titre of 1:100. The IgG-binding test was performed in triplicate and each sample was tested in duplicate. The results were expressed as a mean value of IgG-binding activity. The positive control was a serum with a known titre of 1:1000. The negative control was a serum with a known titre of 1:100.

On 10 January 2013, the European Commission issued a communication entitled 'EU-India: Towards a strategic partnership' (hereinafter referred to as 'the Communication').<sup>1</sup> The Communication sets out the EU's vision for its relationship with India, which it sees as a 'strategic partner'. It highlights the importance of India's role in the world and its potential to contribute to global stability and prosperity. The Communication also outlines the EU's priorities for cooperation with India across various sectors, including trade, investment, climate change, democracy, and development.

No. 11151.

# Immunohistochemistry of a prostate membrane specific protein during development and maturation of the human prostate

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## ABSTRACT

An antiserum against secretory vesicles from human seminal fluid (prostasomes) was used to study the localisation and distribution of the respective antigen(s) during prenatal development and pubertal maturation of the human prostate. The crude antiserum stained both secretory and membrane proteins in the adult prostate and other glands, such as pancreas and parotid gland. An immunoaffinity purified fraction from the antiserum selectively reacted with the apical plasma membrane of prostatic epithelium adluminal cells, recognizing a 100 kDa antigen (PMS). Even in the earliest stages of embryonic prostate specimens studied, the adluminal plasma membrane of the epithelial cells from developing glandular anlagen reacted strongly. The occurrence of PMS immunoreactivity in prostatic anlagen was directly correlated with lumen formation. As the antigen is an androgen-independently synthesised membrane protein of the prostate, it may possibly be used as a marker of cell polarity in the normal and pathologically altered prostate.

**Key words:** Prostate membrane specific protein; prostate development.

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## INTRODUCTION

The human prostate is derived from the upper part of the urogenital sinus in the region into which the mesonephric and paramesonephric ducts open (Cunha, 1994). During the 7th wk of gestation, male embryos develop a colliculus seminalis in the cranial part of the urethra (Kellokumpu-Lehtinen et al. 1980). By the 9th wk, the epithelium covering the colliculus consists of a monolayer of columnar cells. Prostatic development then starts with proliferation of the mesenchyme surrounding the urogenital sinus. At the age of 10 wk, when the verumontanum has developed, histological differentiation begins close to the openings of the mesonephric ducts by outgrowth of several buds of the urethral epithelium into the surrounding mesenchyme. Between the 11th and 14th wk, some of the buds have acquired a lumen and they start to transform into tubular-acinar anlagen (Kellokumpu-Lehtinen et al. 1980). This process proceeds until birth, when in the perinatal period most

of the periurethral ducts are filled with squamous metaplastic epithelium.

In human prostatic development, different cell types sequentially appear and disappear, especially in prostatic epithelium and to a lesser degree in prostatic stroma (Aumüller et al. 1994). Cells derived from urogenital sinus epithelium, containing characteristic masses of glycogen, transform into undifferentiated squamous epithelial cells. Immediately after the formation of a lumen inside the initially solid epithelial buds sprouting into the mesenchyme, the adluminal cells differentiate into polarised columnar cells, whereas undifferentiated cells remain in the basal layer which eventually form typical triangular basal cells and neuroendocrine cells.

Whereas the neuroendocrine cells can easily be recognised immunohistochemically by their content of chromogranin and different hormones (de Mesy Jensen & di Sant'Agnese, 1994), the future secretory, adluminal cells are difficult to characterise aside from their location, in that they are nearly completely

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devoid of secretory material, at least during embryonic development. The present study was performed (1) to test the cell specificity of an antibody directed against prostate derived membrane particles (so-called prostasomes), (2) to trace the formation, development and maturation of secretory acinar cells and (3) establish the potential application of the antibody as a marker for prostatic differentiation, both during normal development and malignant transformation.

#### MATERIAL AND METHODS

##### *Antiserum*

Human prostasomes were isolated from semen samples obtained from voluntary semen donors with normal fertility parameters (courtesy of Prof. Krause, Department of Andrology, University Hospital Marburg), using the method of Ronquist & Brody (1985) with minor modifications. After semen liquefaction ( $\sim 45$  min), seminal fluid was separated from spermatozoa by centrifugation at 1000 g for 20 °C. The supernatant was diluted 1:5 with 30 mM Tris/HCl-buffer, pH 7.5 containing 130 mM NaCl (used as a diluent buffer in all further steps) and was ultracentrifuged for 2 h at 4 °C and 105000 g. The pellet was resuspended in 1.0–1.5 ml of the buffer and further purified on a 40 ml Sephadryl S-500 HR column. The resulting purified prostasomes were then resuspended in 1 ml of diluent buffer. Immunisation of female rabbits of the New Zealand strain was undertaken by injecting a mixture of native prostasomes (150 µg of protein) and Gerbu 100 adjuvant solution (Gerbu Biotechnik, Gaiberg, Germany) subcutaneously into the back skin. Three booster injections were given during the following 12 wk. At 10 d after the last injection, blood was collected from the ear vein of the rabbit. After clotting (4 °C, 12 h), the antiserum was obtained by centrifugation for 20 min at 1500 g. The antiserum serum was characterised by Western blotting, using freshly prepared prostasomes as antigen. Antisera against PSA (MAS 343 cf, clone 8, Sera-lab, Crawley Down, England) and acid phosphatase (own polyclonal antibody, Aumüller et al. 1983) were used as reference standards. Details of the production and characterization of the antiserum against prostasome-derived prostate membrane specific antigen (PMS) are to be published elsewhere (Renneberg et al. 1996).

##### *Prostate specimens*

Organ specimens were taken from the files of the Department of Anatomy and Cell Biology, Marburg,

and the Departments of Pathology of the University Hospitals of Marburg (courtesy of Prof. Thomas), Freiburg (courtesy of Prof. Böhm) and Homburg/Saar (courtesy of Prof. Dr Bonkhoff). Specimens had been fixed in neutral formalin during routine autopsies. The following age stages were available: fetuses from the 15, 17, 19, 20–30, 36, 39 and 40 wk of gestation, prostates from 1 newborn, 3.5-mo-old and 3, 4, 5, 14, 15 and 16-y-old boys each, and 2 17-y-old, 1 18 and 1 19-y-old males. All specimens were paraffin-wax embedded. Sections were cut at 5–6 µm thickness, mounted on chromalum-gelatin subbed glass slides and used for immunohistochemistry after removal of paraffin in xylene and ethanol.

##### *Immunohistochemistry*

The unlabelled antibody enzyme method (Sternberger et al. 1970) was used for the polyclonal rabbit antibodies and the indirect immunoperoxidase method for the monoclonal PSA antibody. Polyclonal antibodies against prostasomes (Renneberg et al. 1996) and acid phosphatase, respectively, were applied to deparaffinised sections in a moist chamber at room temperature for 1 h, diluted 1:100 in phosphate buffered saline (PBS). After thorough rinsing of the sections in PBS, they were incubated for 30 min with the secondary antibody (antirabbit IgG, Dianova, Hamburg, Germany, diluted 1:200 in PBS). The soluble peroxidase-antiperoxidase complex (PAP, Dianova, Hamburg, diluted 1:250 in PBS) was added to the sections after thorough rinsing and incubated for 30 min. The immunoreaction was visualised by incubation in 3,3'-diaminobenzidine (DAB, 10 µg) dissolved in 100 ml PBS containing 0.001% hydrogen peroxide (Graham & Karnovsky, 1966). Sections were thoroughly rinsed in PBS and distilled water, dehydrated and mounted in a synthetic resin. For visualisation of the immunoreaction using the monoclonal anti-PSA-antibody (1:20 diluted in PBS, 1 h at room temperature), a secondary peroxidase-conjugated antimouse IgG (Dianova, Hamburg, 1:100 in PBS, 30 min) was applied and the immunoreaction was visualised using the DAB-H<sub>2</sub>O<sub>2</sub> procedure as described. In some cases, nuclear staining with haemalum was performed. After dehydration and mounting in synthetic resin, sections were evaluated in a Zeiss photomicroscope.

For ultrastructural immunolocalization, immersion-fixed (0.1% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.3) BPH specimens were cut into 50–100 µm thick sections using a tissue chopper equipped with a

razor blade. Chopper sections were incubated with the affinity-purified anti-prostasome antiserum (1:10) and subsequently with gold-labelled (5 nm particles) anti-rabbit IgG (1:50). After thorough rinsing in PBS, chopper sections were treated with 1% aqueous osmium solution for 30 min, dehydrated in alcohol and propylene oxide and embedded in Epon. Ultrathin sections were cut at 500 nm on a Reichert ultramicrotome equipped with a diamond knife, briefly stained with uranyl acetate and lead citrate and examined in a Zeiss EM 10 electron microscope. In control incubations, the primary antibody was replaced by 1% swine serum in PBS.

## RESULTS

### *Lumen formation*

The earliest embryonic stages available were from the 15th to the 26th wk of gestation, when the epithelial buds (starting to grow out from the urethra during the 10th wk) have already considerably increased in number, and transform into tubulo-acinar anlagen (Fig. 1a-c). Incipient tubule formation is recorded from the development of a lumen within the initially solid epithelial buds (Fig. 2a). The formation of the lumen during all stages of development is accompanied by the appearance of immunoreactivity for PMS in some centrally located cells (Fig. 2a). Usually 2 or 3 cells abutting each other start elongating, moving their nuclei into the direction of the basement membrane and show a positive immunoreaction at their opposite pole. Polarisation of the cells and appearance of PMS are obviously intimately corre-

lated. Shortly after polarisation of the cells, the appearance of a lumen becomes visible. The underlying 3–5 cell layers are definitely nonreactive with the very rare exception that most basal cells residing on the basement membrane develop some transient immunoreactivity at their basal plasma membrane. The adluminal cells divide frequently, thereby increasing the diameter of the lumen and reducing the height of the stratified epithelial layer. Conversely, at the tips of the solid buds, the basally located cells divide. This results in further piercing of the epithelial buds into the surrounding mesenchymal stroma. The latter acquires a more differentiated structure at the periphery, resembling a capsule which may be responsible for the termination of epithelial budding. The process of lumen formation proceeds in the same way as already described. In embryonic specimens, immunoreactivity for PSA or acid phosphatase was never observed in adluminal PMS-immunoreactive or any other epithelial and neuroendocrine cells.

### *Metaplasia*

Shortly after birth, the larger ducts surrounding the urethra, resume proliferative activity of the basal and intermediate cells, developing a pronounced squamous metaplasia (Fig. 2b, c). Squamous metaplastic cells are removed by simple desquamation into the lumen (Fig. 2b), where they condense and form thick masses of detritus. Within the thick layers of metaplastic epithelium, lumen formation occurs, with differentiation of adluminal cells containing apical PMS immunoreactivity. Sometimes very extensive

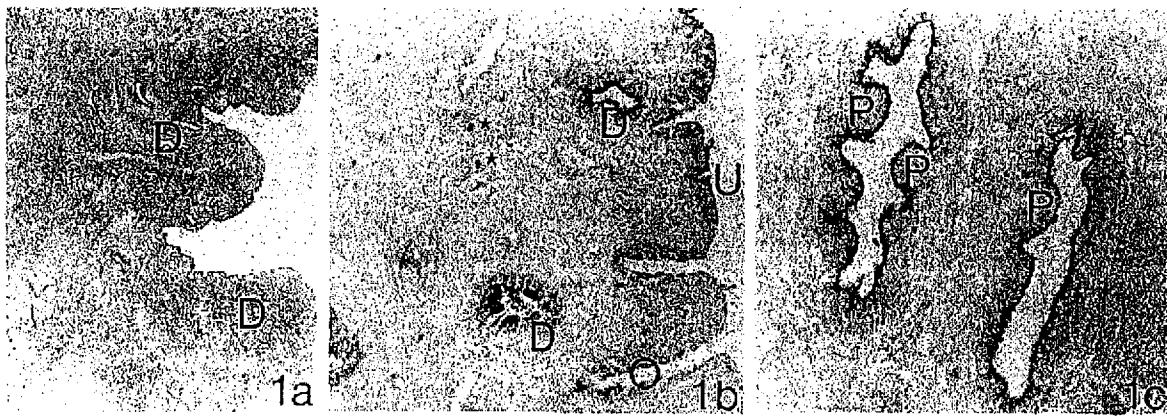


Fig. 1. Immunoreaction of the PMS antiserum in the prostate of an embryo from the 26th wk of gestation. (a) Survey of the prostatic anlagen with strongly labelled large ducts (D).  $\times 2$ . (b) Lateral periurethral budding zone. Note the irregular and moderate staining of the urethral epithelium (U). Homogeneous immunoreactivity is present only in prostatic ducts (D) somewhat distant of their urethral orifices (O). A few immunoreactive macrophages (asterisks) are seen in the stroma.  $\times 20$ . (c) Large ducts from the collicular portion of the same specimen showing primary papillae (P) covered with immunoreactive epithelium.  $\times 20$ .

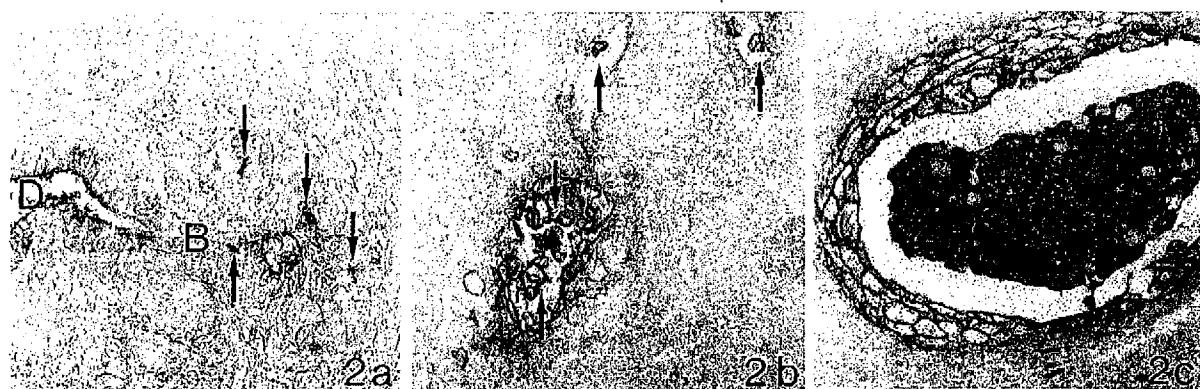


Fig. 2. Incipient lumen formation and focal metaplasia in the prostate of an embryo from the 36th wk of gestation. (a) Spotwise distribution of PMS immunoreactive material (arrows) indicating incipient lumen formation in a solid epithelial prostatic bud (B) originating from a small peripheral duct (D). The adluminal cells of the duct are strongly immunoreactive.  $\times 50$ . (b) Intermediate portion of the same specimen showing desquamating immunoreactive cells (arrows) within the lumen.  $\times 50$ . (c) Periurethral duct with immunoreactive squamous metaplastic epithelium and condensed intraluminal detritus.  $\times 30$ .

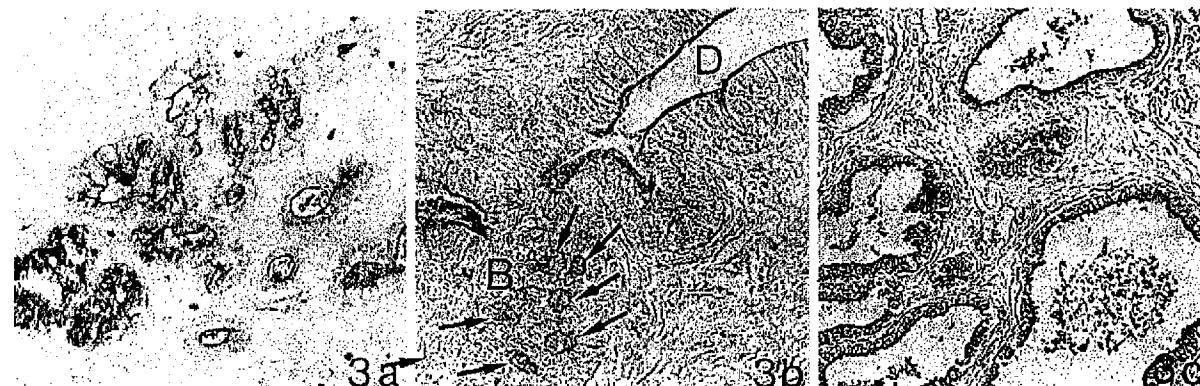


Fig. 3. Distribution of PMS immunoreactivity during postnatal development of the prostate. (a) Central portion of the prostate from a 3.5-mo-old child showing a group of ducts, some of which contain metaplastic immunoreactive epithelium and newly formed ducts with immunoreactive adluminal cells.  $\times 20$ . (b) Prostate of a 4-y-old boy with forming lumina (arrows) inside a solid cell mass (B) adjacent to a large duct (D) close to the urethral opening.  $\times 5.2$ . (c) Maturing prostate from a 16-y-old boy. Peripheral prostatic acini covered with strongly immunoreactive adluminal cells.  $\times 5.3$ .

lumen formation renders the multilayered epithelium a cribriform structure (Fig. 3a, b). The basal cells and the neuroendocrine cells never show PMS immunoreactivity on their lateral or basal surfaces.

#### Cell specificity

In prostatic ducts, mostly in their intermediate segments, individual PMS-positive cells start developing both PSA and acid phosphatase immunoreactivity (not shown). Also, the most peripherally located PAS-positive cells (Aumüller et al. 1994) which generally lack PSA or acid phosphatase immunoreactivity, contain an adluminal rim of PMS immunoreactivity (not shown).

Postnatal development of the prostate is most appropriately divided into 3 different phases (Swyer, 1944; Andrews, 1951; Aumüller, 1979): (1) a perinatal

and a subsequent regression phase, (2) an infantile resting period and (3) the pubertal maturation period. During the regression and the resting periods no major deviations from the perinatal situation is recorded with regard to PMS-immunoreactivity (Fig. 3a). During puberty the general development of the ductal epithelium with basal and adluminal secretory cells spreads throughout the gland, starting at about 11–12 y. An increase in the diameter of the lumen of the ducts occurs that is readily recognised from the changing pattern of PMS-immunoreactivity of the developing acini. This is accompanied by the formation of secondary and tertiary ramifications of the glandular ducts, pushing the interstitial stroma aside. At the same time, removal of any remnants of squamous metaplastic epithelium is completed (Fig. 2b), and the number of the PAS-positive cells decreases significantly (not shown).

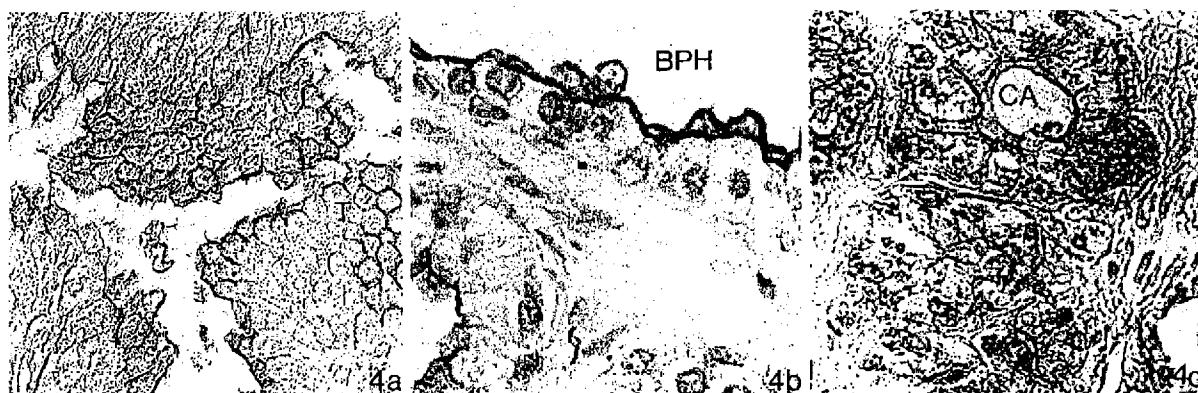


Fig. 4. High power magnification of the labelling distribution in the normal (*a*, *b*) and malignant (anaplastic) prostate. (*a*) Slightly oblique section through apical cell poles (T) showing the labelling of the circumferences of apical plasma membranes.  $\times 200$ . (*b*) Atrophic epithelium in a case of benign prostatic hyperplasia (BPH) with strongly labelled apical plasma membrane; nuclei counterstained with haemalum.  $\times 750$ . (*c*) Differentiated prostate cancer with membrane-labelling of gland-like structures (CA). In an adjacent anaplastic portion (A) generalised cytoplasmic labelling is seen; nuclei are slightly counterstained with haemalum.  $\times 600$ .

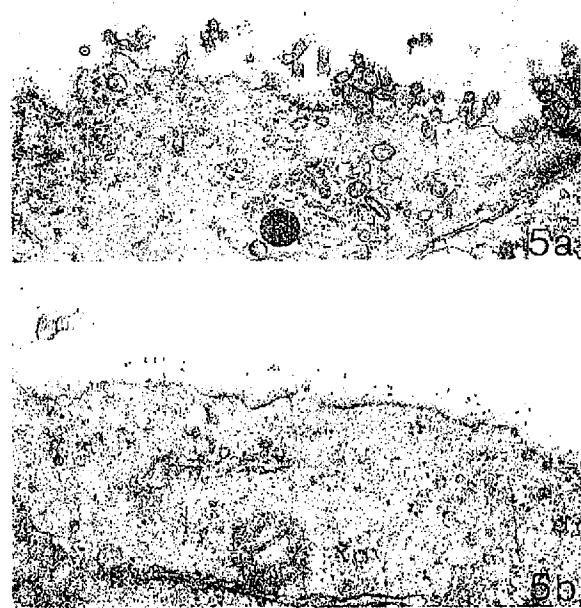


Fig. 5. Immunoelectron microscopy of PMS immunoreactivity in the adult prostate. (*a*) Immunogold particles are concentrated on the apical plasma membrane of a secretory cell covered by microvilli.  $\times 40000$ . (*b*) Labelling of the tangentially cut smooth surface of a prostatic secretory cell.  $\times 40000$ .

The formation of mature acini starts at about 15–16 y and can be traced by the changing pattern of PMS immunoreactivity and increasing PSA or acid phosphatase immunoreactivity. Whereas in the large periurethral ducts, which usually contain a multi-layered epithelium until the age of 15–16 y, admixed cells containing secretory material are interspersed with nonreactive cells, the intermediate duct portion

forms secondary and tertiary ramifications, which gradually distend, forming sacculations separated by papillae (made of connective tissue and smooth muscle cells). The peripheral subcapsular acini are small, round or have few sacculations and their epithelium consists of cells nonreactive for secretory proteins, but have distinct PMS immunoreactivity at their apical plasma membrane.

#### Cell polarity

In the mature prostate the highly purified 100 kDa antibody reacts strongly at the apical plasma membrane (Figs 3*c*; 4*a, b*). At the electron microscopic level the exclusive labelling of the outer leaflet of the apical plasma membrane becomes evident (Fig 5*a, b*). This pattern of immunoreactivity is retained in well differentiated prostate cancer, but lost in anaplastic cancer cells (Fig 4*c*), where all of the cytoplasm stains or stain is completely lost.

#### DISCUSSION

##### *Characteristics of the antiserum*

In the present study we have used an antiserum raised against prostasomes (Ronquist & Brody, 1985), i.e. particles that were thought to be released from the prostate during ejaculation. Western blotting experiments clearly confirmed the composite variety of antibodies present in the crude antiserum among which, however, one antigen of about 100 kDa was prevalent. Immunoaffinity purification of the relevant antibody (anti-100 kDa) resulted in a highly specific labelling exclusively of the apical plasma membrane of prostatic secretory cells.

The question, therefore, is to which of the known prostatic antigens the 100 kDa antigen would be related and what its nature, prostate specificity and androgen dependence would be. The most likely candidate responsible for the 100 kDa band detected by our immunopurified antibody is the so-called prostate membrane specific antigen (PSM), recently described by Israeli et al. (1993, 1994). Israeli et al. (1994) found the highest PSM expression in hormone-deprived states of the prostate. In benign prostatic hyperplasia, PSM was at times absent. All these features are very much in favour for the assumption that PSM is related to or identical with our 100 kDa antigen present in the apical plasma membrane of prostatic adluminal cells. The respective antibody, therefore, should be well suited for the study of prostatic development.

#### *Organogenesis and maturation of the prostate*

The prostate derives from a composite area of the urogenital sinus (Glenister, 1962) and is thought to require both oestrogens and androgens during organogenesis (Neumann et al. 1974; Pylkänen et al. 1992).

The formal development of the human prostate has been thoroughly studied both at the light and electron microscopic level, e.g. by Kellokumpu-Lehtinen et al. (1980). The 100 kDa antibody used in the present study has the advantage that it easily recognises lumen formation. The immunoreactive adluminal cell layer is the first cell type showing an advancing step of differentiation relative to the basal cells. The 100 kDa antibody, therefore, may serve as a marker of initial prostatic differentiation. A second step of differentiation would then be the androgen-dependent expression of secretory proteins within such cells which are already immunoreactive for the 100 kDa antigen.

Lumen formation is an essential step during glandular organogenesis and is obviously a highly regulated and genetically fixed process which is also retained after malignant transformation. Interestingly, the patterns of lumen formation and development observed during organogenesis are partly reflected in prostate cancer, ranging from the glandular to the cribriform pattern.

Another important aspect in the expression of the 100 kDa antigen is its occurrence during an androgen-deficient state, at least during a period where androgen levels inside the prostate are very low, contrary to the oestrogen levels (Zondek et al. 1986). Whereas the secretory proteins are immunohistochemically

detected only after the onset of puberty (although faint immunoreactivity may be present in the glands of young children), the 100 kDa antigen is present adluminally from the very beginning. This would perhaps indicate that this antigen would be retained in prostate cancer cells, even when they have lost their androgen dependence. The PMS antigen is retained more homogeneously in prostate cancer cells relative to PSA. The only significant deviation from normal cells observed was the increased cytoplasmic staining in anaplastic cells compared with the apical membrane staining in intact cells. In other words, the changed distribution of the antigen in anaplastic cells would indicate a loss of polarity of these cells due to disturbed membrane trafficking.

In conclusion, the prostate membrane specific protein present in human seminal/prostasomal proteins has been detected immunohistochemically on the apical plasma membrane of prostatic adluminal cells. Its unique localisation, already evident during the earliest stages of development, shows that it is synthesised in an androgen-independent manner, which is in contrast to the ordinary secretory proteins of the gland. It may be used as a marker of growth patterns in the normal and pathologically altered prostate.

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## Prostate Secretory Protein/PSP antibody [YPSP-1] (ab19070) datasheet

<b>Product Name</b>	Prostate Secretory Protein/PSP antibody [YPSP-1]
<b>Product type</b>	Primary antibodies
<b>Description</b>	Mouse monoclonal [YPSP-1] to Prostate Secretory Protein/PSP
<b>Immunogen</b>	Full length protein (purified) (Human).
<b>Reacts with (species key)</b>	Hu
<b>Specificity</b>	Does not react with a variety of human normal tissues.
<b>Tested applications</b>	ELISA, Western blot

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### Application notes

(see key)

### Recommended dilutions

ELISA: Use at an assay dependent dilution.

WB: Use at an assay dependent dilution. Predicted molecular weight: 14 kDa.

Not tested in other applications.

Optimal dilutions/concentrations should be determined by the end user.

Reactive with PSP or human prostate epithelial cells.

### Positive control (see definition)

Secreted. Sperm surface.

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### Research areas

An abundant constituent of the human seminal plasma is a 14-kD protein of prostate origin known as beta-microseminoprotein, prostatic secretory protein (PRPS), Prostate secretory protein (PSP), or PSP94. It is a product of a single gene transcribed in the prostate but not in the testis. Specific receptors for this protein are found on spermatozoa and in the prostate.

### Database links

<b>GeneCard</b>	<a href="#">GC10P051219</a> (Human)	<b>SwissProt</b>	<a href="#">P08118</a> (Human)
<b>Omim</b>	<a href="#">157145</a> (Human)	<b>Unigene</b>	<a href="#">255462</a> (Human)

### Raised in

Mouse

### Clonality

Monoclonal

### Clone number

YPSP-1

### Isotype

IgG1

Purity	Protein G purified
Storage buffer	Preservative: None Constituents: 0.01M PBS, pH 7.2
Form	Liquid
Concentration	1.000 mg/ml
Storage instructions	Aliquot and store at -20°C. Avoid freeze / thaw cycles.

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Presentation: Protein G purified antibody in 10mM PBS with 0.2% BSA and 15mM NaN3. (Bulk available without BSA/Azide upon request) concentration 200ug/ml

Immunogen: purified human PSA

Isotype: mIgG1 (both)

Clones: ER-PR8 & PA05

Reactivity: human(others not tested)

Uses: -Western blot: 1ug/ml 2 hr rt

-histochemistry (frozen and paraffin sections) approx 1-2ug/ml 30-60 minutes RT

-for formalin fixed sections, requires pretreatment in boiling 10mM citrate bufer pH6.0 for 10-20 minutes followed by cooling at RT for 20 min.

Positive Contrl: prostate or prostate carcinoma

Localization: Cytoplasmic

**Background:** Recognizes a single protein of 33-34kDa, identified as PSA. This "cocktail" is composed of two clones those epitopes are not precisely mapped, but experimental data suggests that they are different. This cocktail is highly specific to PSA and stains prostatic secretory and ductal epithelium in both normal and neoplastic tissues. PSA is a chymotrypsin-like serine protease (kallikrein family) exclusively produced by the prostate epithelium, and abundant in seminal fluid. PSA can be detected in the sera of patients with prostatic carcinoma. It is predominantly complexed to a liver derived serine protease inhibitor, alpha-1- antichymotrypsin. A higher proportion of serum PSA is complexed to ACT in prostate cancer than in benign prostate hyperplasia.

Storage: 2-8 deg c

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Preparation: Lyophilized antibody purified by Protein G affinity chromatography diluted in 1% w/v BSA in PBS with 15mM sodium azide. Reconstitute with 1ml of sterile distilled water.

Clone: PSA 28/A4

### Antigen: seminal fluid preparation

Isotype: mIgG1k

**Specificity:** Human prostate specific antigen (this antibody reacts with prostatic secretory and ductal epithelium in both normal and neoplastic tissue and can be useful in the identification of metastatic carcinomas of prostatic origin)

USE: Effective on paraffin wax embedded tissue and on frozen sections.

Protocol: Immunohistochemistry: Typical working dilution 1:50 - 1:100. 60 Minutes incubation at 25°C.  
Standard ABC technique.

Positive controls: normal prostate gland or known prostate carcinoma

Staining pattern: cytoplasmic

**Storage and stability:** Store unopened lyophilized antibody at 4°C. Under these conditions, there is no significant loss in product performance up to the expiry date indicated on the vial label. The reconstituted antibody is stable for at least two months when stored at 4°C. For long term storage, aliquot in non frost freeze freezer, Avoid repeated freeze thaw cycles. Prepare fresh working dilutions daily. Strictly for in vitro research use only-Not for use in or on humans or animals-not for use in diagnostics

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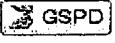
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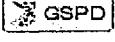
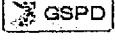
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## PATE (Prostate and testis expressed gene) Antibody Search

### PATE (Prostate and testis expressed gene) Antibodies

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Reactivity

Species with which the antibody reacts

Any

**Host Species**

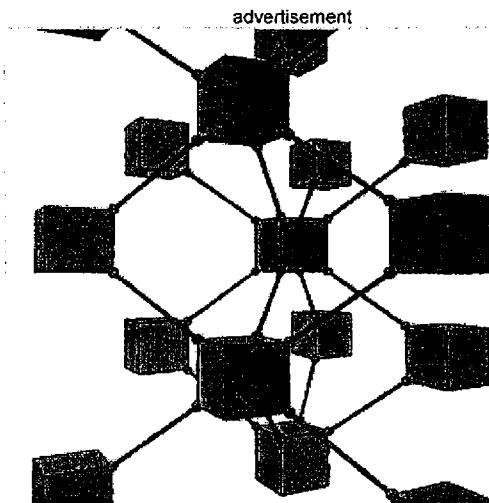
Host in which the antibody was generated

 Any**Applications**

Specific antibody applications

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Clone: 2H9, 5A6, 8A6, PS1, PS2 New clones - PS6, 5G6

These clones have been derived from hybridization of SP2/0 myeloma cells with spleen cells of a Balb/c mice immunized with human PSA antigen purified from seminal plasma.

Reactivity: Specificity of the clone is described in the table (see below)

Application: Detection of PSA antigen.

Recommended pairs for detection of total PSA : 5A6-2H9, PS2 - PS6, 5G6 - 5A6.

**Best pair : 5G6 - 5A6**

Recommended pairs for detection of free PSA : 5A6-8A6, 2H9-8A6, PS2-PS1, PS1-PS2, 8A6 - 5A6

**Best pair : 8A6 - 5A6**

Purification: Purified by chromatography on protein A Sepharose. Purity is tested by electrophoresis.

Presentation: Each vial contains antibodies in PBS, pH 7,4, containing 0,1% of sodium azide as preservative. Store at +4C.

Material safety note: This product is sold as an antibody preparation for research purpose only. Standard Laboratory Practices should be followed when handling this material. Contains sodium azide (0,1%) as preservative. Although the amount of sodium azide is very small appropriate care must be taken when handling this product.

Mab	Isotype	Specificity in ELISA	Cross-reactivity with human kallikrein 2
5G6	IgG1	Total PSA, epitope 4	0.00
PS6	IgG1	Total PSA, epitope 4	0.00
2H9	IgG2a	equimolar total PSA, epitope 3.	50%
PS2	IgG1	equimolar total PSA, epitope 3	100%
5A6	IgG2a	equimolar total PSA epitope 6	0.00
8A6	IgG2a	free PSA epitope 1	0.00
PS1	IgG1	free PSA, epitope 1	0.00

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**Mouse anti-Human PSA MONOCLONAL**

cat# RDI-PSAabm-280 \$375.00/1mg

Clone# 280

Lot# see batch sheet

Use: Coating antibody for solid phase

Class: mouse IgG

Antigen: human seminal fluid

Presentation: Chromatographically purified immunoglobulin fraction in 0.05M PBS, pH 7.4 containing 0.1% NaN<sub>3</sub>.

Volume: see batch sheet

Protein conc: see batch sheet

Affinity: 8 X 10<sup>9</sup>

Storage: Store at 4-8 DEG C Upon Receipt. Avoid Frequent freeze thaw cycles.

For IN VITRO RESEARCH OR FURTHER MANUFACTURING USE ONLY.

Price: Price: \$375.00/mg 1-9mg    \$312.00/mg 10mg +    Bulk on request

---

#### **Mouse anti-Human PSA MONOCLONAL (Total-detecting ab)**

cat: RDI-PSAabm-281      Price: Price: Price: \$375.00/mg 1-9mg    \$344.00/mg 10mg +

Bulk on request

Clone# 281

Lot# see batch sheet

Use: Detecting antibody (enzyme or <sup>125</sup>I labeled )

Class: mouse IgG

Antigen: human seminal fluid

Presentation: Chromatographically purified immunoglobulin fraction in 0.05M PBS, pH 7.4 containing 0.1% NaN<sub>3</sub>.

Volume: see batch sheet

Protein conc: see batch sheet

Affinity: 6 X 10 E09

Storage: Store at 4 -8 DEG C Upon Receipt.

also: directly conjugated **anti-PSA:HRP** monoclonal clone 281

cat#RDI-PSAabm-HRP \$625.00/milligram

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### **Mouse anti-Human FREE PSA MONOCLONAL**

cat: RDI-PSAFabm-65 Price: \$500.00/mg 1-5mg \$438.00/mg 6-20mg

Clone# PSA-F65

Lot# see batch sheet

Use: Coating antibody (pair with clone 66 )

-for use in EIA or IRMA assays

Class: mouse IgG1

Antigen: purified human Free PSA

Presentation: Chromatographically purified immunoglobulin fraction in PBS, pH 7.5 containing 0.1% NaN3.

Volume: see batch sheet

Protein conc: see batch sheet

Affinity: approx 2 X 10 E10

Specificity: Free PSA 100% less than 0.1% with AFP, CEA, Ca 19-9, CA125, PAP, albumin, human IgG)

Storage: Store at 4 -8 DEG C Upon Receipt.

For IN VITRO RESEARCH OR FURTHER MANUFACTURING USE ONLY.

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### **Mouse anti-Human PSA MONOCLONAL (Detecting clone in FREE PSA assay)**

cat: RDI-PSAabm-66 Price: \$500.00/mg 1-5mg \$438.00/mg 6-20mg

Clone# PSA-66

Lot# see batch sheet

Use: Detecting antibody (pair with free PSA clone 65)

-for use in EIA or IRMA assays

Class: mouse IgG1

Antigen: purified human PSA

Presentation: Chromatographically purified immunoglobulin fraction in PBS, pH 7.5 containing 0.1% NaN3.

Volume: see batch sheet

Protein conc: see batch sheet

Affinity: approx 7 X 109

Specificity: PSA 100% less than 0.1% with AFP, CEA, CA 19-9, CA125, PAP, albumin, human IgG)

Storage: Store at 4 -8 DEG C Upon Receipt.

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## Prostate Specific Antigen antibody [PSA1] (ab403) datasheet

<b>Product Name</b>	Prostate Specific Antigen antibody [PSA1]				
<b>Product type</b>	Primary antibodies				
<b>Description</b>	Mouse monoclonal [PSA1] to Prostate Specific Antigen				
<b>Immunogen</b>	Full length native protein (purified) (Human).				
<b>Reacts with (species key)</b>	Hu				
<b>Specificity</b>	<p>Human prostate-specific antigen - 100%</p> <p>PSA/alpha-1-ACT-complex - 59%</p> <p>Kallikrein - &lt;0.04%</p> <p>PAP - &lt;0.04%</p> <p>Cross-reactivity values have been calculated on weight/weight basis using 125-I-labelled human human prostate-specific antigen in assay. They can vary because of the presence of antigen or related impurities in protein preparations used for cross reactivity assays.</p>				
<b>Tested applications</b>	Affinity constant: $1 \times 10^{11}$ l/mol human prostate-specific antigen. Radioimmunoassay, ELISA				
<b>Abreviews</b> <small>Reviews by Abcam customers (see key)</small>	<p><a href="#">Submit an Abreview</a> and earn up to 120 Abpoints by reviewing this product</p> <table border="0"> <tr> <td>• IHC-P</td> <td><a href="#">1 Abreview</a></td> </tr> <tr> <td>• WB</td> <td><a href="#">1 Abreview</a></td> </tr> </table> <p><a href="#">(Read all Abreviews)</a></p>	• IHC-P	<a href="#">1 Abreview</a>	• WB	<a href="#">1 Abreview</a>
• IHC-P	<a href="#">1 Abreview</a>				
• WB	<a href="#">1 Abreview</a>				
<b>Application notes (see key)</b>	<p><b>Recommended dilutions</b></p> <p>ELISA: Use at an assay dependant concentration.</p> <p>RIA: Use at an assay dependant concentration.</p> <p>Nanowires array: 1/50 - 1/500.</p> <p>Not tested in other applications.</p> <p>Optimal dilutions/concentrations should be determined by the end user.</p>				
<b>Cellular localization</b>	Cytoplasmic and Secreted				
<b>Research areas</b>	<p><a href="#">Cancer</a> &gt;&gt; <a href="#">Tumor biomarkers</a> &gt;&gt; <a href="#">Tumor antigens</a></p> <p><a href="#">Cancer</a> &gt;&gt; <a href="#">Tumor immunology</a> &gt;&gt; <a href="#">Tumor-associated antigens</a></p> <p><a href="#">Cell Biology</a> &gt;&gt; <a href="#">Cytoskeleton / ECM</a> &gt;&gt; <a href="#">Extracellular Matrix</a> &gt;&gt; <a href="#">ECM Enzymes</a></p> <p>&gt;&gt; <a href="#">Kallikreins</a></p> <p><a href="#">Tags &amp; Cell Markers</a> &gt;&gt; <a href="#">Cell Type Markers</a> &gt;&gt; <a href="#">Tumor Associated</a></p> <p><a href="#">Neuroscience</a> &gt;&gt; <a href="#">Cell Adhesion Proteins</a> &gt;&gt; <a href="#">Proteases</a></p>				
<b>Relevance</b>	Kallikreins are a subgroup of serine proteases having diverse physiological functions. Growing evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel cancer and other disease biomarkers. This gene is one of the fifteen kallikrein subfamily members located in a cluster on chromosome 19. Its protein product is a protease				

present in seminal plasma. It is thought to function normally in the liquefaction of seminal coagulum, presumably by hydrolysis of the high molecular mass seminal vesicle protein. Serum level of this protein, called PSA in the clinical setting, is useful in the diagnosis and monitoring of prostatic carcinoma.

Alternate splicing of this gene generates two transcript variants encoding different isoforms. Additional transcript variants have been described, but it is unclear if these transcripts are normally expressed or if they are specific to benign or malignant tumors.

<b>Database links</b>	<b>Entrez Gene</b>	354 (Human)	<b>SwissProt</b>	P07288 (Human)
	<b>GeneCard</b>	GC19P056049 (Human)	<b>Unigene</b>	171995 (Human)
	<b>Omim</b>	176820 (Human)		
<b>Raised in</b>	Mouse			
<b>Clonality</b>	Monoclonal			
<b>Clone number</b>	PSA1			
<b>Isotype</b>	IgG1			
<b>Purity</b>	Protein A purified			
<b>Storage buffer</b>	Preservative: 0.1% Sodium Azide Constituents: Sodium Chloride, Citrate / phosphate. pH 6.0			
	Material safety datasheet (MSDS) for this product: <a href="#">Sodium Azide MSDS</a>			
<b>Form</b>	Liquid			
<b>Concentration</b>	5.300 mg/ml			
<b>Storage instructions</b>	Store at +4°C.			

At Abcam, we have one centralized database to hold all of our product information, so that everything we know about this Prostate Specific Antigen antibody [PSA1] is on this datasheet. But please do [contact us](#) if you would like any reassurance!



## References for Prostate Specific Antigen antibody [PSA1] (ab403)

### This product has been used in:

Zheng G et al. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. *Nat Biotechnol* 23:1294-301 (2005). [PubMed: 16170313](#)

If you publish research using ab403 please [let us know](#) so that we can cite the reference on this datasheet.

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**Prostate Specific Antigen antibody [PSA1] - related products:****Compatible Secondaries for Prostate Specific Antigen antibody [PSA1]**

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ab6563	<u>Cy5 ® antibody</u>
ab6726	<u>Texas Red ® antibody</u>
ab6728	<u>HRP antibody</u>
ab6729	<u>Alkaline Phosphatase antibody</u>
ab6785	<u>FITC antibody</u>
ab6788	<u>Biotin antibody</u>
ab7002	<u>Phycoerythrin antibody</u>
ab7018	<u>Agarose antibody</u>
ab27229	<u>15nm Gold antibody</u>
ab27230	<u>10nm Gold antibody</u>
ab27232	<u>20nm Gold antibody</u>
ab27233	<u>5nm Gold antibody</u>
ab27241	<u>10nm Gold antibody</u>
ab27242	<u>20nm Gold antibody</u>
ab27243	<u>40nm Gold antibody</u>
ab27244	<u>5nm Gold antibody</u>

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## Identification of Novel Human CTL Epitopes and Their Agonist Epitopes of Mesothelin

Junko Yokokawa,<sup>1</sup> Claudia Palena,<sup>1</sup> Philip Arlen,<sup>1</sup> Raffit Hassan,<sup>2</sup> Mitchell Ho,<sup>2</sup> Ira Pastan,<sup>2</sup> Jeffrey Schlom,<sup>1</sup> and Kwong Y. Tsang<sup>1</sup>

**Abstract** **Purpose:** Mesothelin is overexpressed in many pancreatic and ovarian cancers, mesotheliomas, and other tumor types. Clinical trials are ongoing using immunotoxins to target mesothelin, and patients immunized with allogeneic pancreatic tumor cell lines have shown immune responses to previously defined mesothelin epitopes. The purpose of this study was to define novel mesothelin CTL epitopes and, more importantly, agonist epitopes that would more efficiently activate human T cells to more efficiently lyse human tumors.

**Experimental Design and Results:** Two novel mesothelin HLA-A2 epitopes were defined. T-cell lines generated from one of these epitopes were shown to lyse pancreatic and ovarian tumor cells. Several agonist epitopes were defined and were shown to (a) have higher affinity and avidity for HLA-A2, (b) activate mesothelin-specific T cells from normal individuals or cancer patients to a greater degree than the native epitope in terms of induction of higher levels of IFN- $\gamma$  and the chemokine lymphotactin, and (c) lyse several mesothelin-expressing tumor types in a MHC-restricted manner more effectively than T cells generated using the native peptide. External beam radiation of tumor cells at nontoxic levels was shown to enhance the expression of mesothelin and other accessory molecules, resulting in a modest but statistically significant increase in tumor cell lysis by mesothelin-specific T cells.

**Conclusions:** The identification of novel CTL agonist epitopes supports and extends observations that mesothelin is a potential target for immunotherapy of pancreatic and ovarian cancers, as well as mesotheliomas.

Mesothelin, a glycosylphosphatidylinositol-linked glycoprotein, is a differentiation antigen of mesothelial cells. The cDNA encodes a  $M_r$  69,000 precursor protein, which is proteolytically processed into two components. One component, corresponding to the COOH-terminal portion of the precursor, is a membrane-bound  $M_r$  40,000 protein known as mesothelin. The other component is a  $M_r$  30,000 protein, corresponding to the NH<sub>2</sub>-terminal-secreted protein megakaryocyte potentiating factor (1–3). In humans, mesothelin is expressed in normal mesothelium and certain epithelial cells of the tonsil, trachea, and fallopian tube (4). Mesothelin is overexpressed in a vast majority of ductal pancreatic adenocarcinomas; mesotheliomas; and adenocarcinomas of the ovary, lung, and stomach (5–14). Mesothelin has also been reported in squamous cell carcinomas of the esophagus, lung, and cervix (15).

The presence of mesothelin in many cancer cells makes it a potential target for cancer therapies. A monoclonal antibody, K1, which recognizes mesothelin, has been developed and used in preclinical studies (4, 16). Later, a high-affinity antimesothelin antibody was isolated by phage display (17) and after affinity improvement (18) was converted to a disulfide-linked Fv and fused to a 38 kDa portion of *Pseudomonas* exotoxin A to make immunotoxin SS1(dsFv)-PE38 (3). SS1(dsFv)-PE38 selectively inhibited experimental human lung cancer metastases in nude mice as well as showed antitumor activity against tumor cells obtained from patients with ovarian cancer and peritoneal mesotheliomas (19–21). Immunotoxins directed against mesothelin are currently in clinical trials (22, 23).

Mesothelin is also a potential target for T-cell direct immunity. Potential CTL epitopes of mesothelin specific for HLA-A2, HLA-A3, and HLA-A24 have recently been reported. CD8 $^+$  T-cell responses to these HLA-A2, HLA-A3, and HLA-A24 epitopes were detected in patients vaccinated with granulocyte macrophage-colony stimulating factor-transduced pancreatic cancer cell lines (24). These studies provided evidence of cross-priming postvaccination, which was directed against mesothelin-specific epitopes (24).

Several preclinical studies have shown that the modification of CTL epitopes to render them more immunogenic will result in enhanced antitumor responses. Recent clinical studies in melanoma and colorectal cancer have also shown that vaccination with such agonist epitopes can result in the generation of greater levels of T-cell responses in patients, which have

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correlated with clinical responses (25, 26). It has also recently been shown that agonist epitopes have the ability to stimulate T cells to produce more lymphotactin. Lymphotactin is a member of the C-chemokine family cloned from activated prot-T cells (27, 28). It is produced by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, natural killer cells, intraepithelial  $\gamma\delta$  T cells, and mast cells (29–31). Lymphotactin is a powerful chemoattractant for CD4<sup>+</sup> and CD8<sup>+</sup> T cells and a moderate chemoattractant for natural killer cells (28, 30).

The study described here reports the identification and characterization of a novel mesothelin CTL epitope, and the generation of an enhancer agonist of this epitope. T-cell lines, generated from a pancreatic cancer patient with the agonist peptide, showed high levels of lysis of mesothelin-expressing tumor cells and enhanced IFN- $\gamma$  and lymphotactin production when target cells were pulsed with the agonist peptide versus the native peptide. The studies reported here also show that the use of nontoxic doses of external beam radiation of tumor cells will enhance the expression of mesothelin and other accessory molecules and render tumor cells more susceptible to mesothelin-specific T-cell killing. These studies thus provide the rationale for the potential utility of the novel mesothelin agonist epitope, alone or in concert with previously defined mesothelin epitopes, in peptide- and/or vector-mediated immunotherapy protocols for the treatment of mesothelin-expressing tumors.

## Materials and Methods

**Cell cultures.** A human pancreatic adenocarcinoma cell line CFPAC-1 (HLA-A2 positive and mesothelin positive; ref. 32), a human colon carcinoma SW1463 (HLA-A2 positive, mesothelin negative), a human ovarian adenocarcinoma cell line OVCAR-3 (HLA-A2 positive, mesothelin positive), and a human pancreatic adenocarcinoma cell line AsPC-1 (HLA-A2 negative and mesothelin positive) were purchased from American Type Culture Collection (Manassas, VA). Three HLA-A2-positive and mesothelin-positive peritoneal mesothelioma cell lines (YOU, ROB, and ORT), a human epidermoid carcinoma cell line A431 (HLA-A2 negative, mesothelin negative), and A431.H9 cell line were obtained from Dr. Raffit Hassan (National Cancer Institute, NIH, Bethesda, MD). A431 cells transfected with pMH107, pcDNA3.1(+) vector containing a full-length mesothelin cDNA are designated as A431.H9. A431.H9 is a stable transfected cell line (33). The cultures were free of *Mycoplasma* and were maintained in complete medium [RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin (Invitrogen Life Technologies)]. The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or HLA-B antigens (34). C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1 (35). These cells were obtained from Dr. William E. Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). The 174CEM-T2 cell line (T2) transport deletion mutant (36) was provided by Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT). C1R-A2 cells and T2 cells were *Mycoplasma*-free and were maintained in RPMI 1640 complete medium and in Iscove's modified Dulbecco's complete medium (Invitrogen Life Technologies), respectively.

**Peptides.** The amino acid sequence of mesothelin was scanned for matches to consensus motifs for HLA-A2-binding peptides. We used the computer algorithm from the Bioinformatics and Molecule Analysis Section of NIH, developed by Parker et al. (37), which ranks potential MHC-binding peptides according to the predictive one-half-time

dissociation of peptide/MHC complexes. The HLA-A2 allele was chosen because it is the most commonly expressed class I allele. Nine-mer and 10-mer peptides were synthesized if they conformed to the respective consensus motif. A panel of 10-mer mesothelin peptides (Table 1) and analogues with one-, two-, or three-amino acid substitution of P-547-556 peptide and MUC-1 peptide (38) were made by American Peptide Company (Sunnyvale, CA) with purity >90%.

**Flow cytometric analysis.** Dual-color flow cytometric analysis was done on T-cell lines by using the following antibody combinations: anti-CD56-FITC/anti-CD8-PE, anti-CD8-FITC/anti-CD45RA-PE, anti-CD8-FITC/anti-CD27-PE, and anti-CD8-FITC/anti-CD28 PE. Antibodies were all purchased from BD Biosciences (San Jose, CA). Staining was conducted simultaneously for 1 hour at 4°C; cells were then washed thrice with cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS, resuspended in the same buffer, and immediately analyzed using a FACScan and the CELLQuest program (BD Biosciences). Data were gathered from 10,000 live cells, stored, and used to generate results.

The procedure for analysis of dendritic cells was similar to the one described above. The following antibody combinations were used: anti-MHC-class II-FITC/anti-CD80-PE, anti-CD58-FITC/anti-CD54-PE, anti-MHC class I-FITC/anti-MHC class II-PE, and anti-IgG1-FITC/anti-IgG2a-PE (isotype controls). Antibodies to MHC class I and class II were purchased from Serotec (Oxford, United Kingdom); other antibodies were purchased from BD Biosciences. The method described by Fan et al. (19) using K1 antibody was used for the analysis of mesothelin expression on tumor cell lines. The cells were immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 mW at 488 nm. Data were gathered from 10,000 live cells, stored, and used to generate results.

Results were expressed in percentage of positive cells and mean fluorescence intensity. Mean fluorescence intensity was used to express the levels of fluorescence determined by measuring the average for all the cells in the gated fluorescence dot plot. The mean fluorescence intensity value was collected in log scale on the FACScan.

**Peptide binding to HLA-A2.** Binding of mesothelin peptides and the mesothelin analogues to HLA-A2 molecules was evaluated by the up-regulation of HLA-A2 expression on T2 cells as shown by flow cytometry (39).

**Culture of dendritic cells from peripheral blood mononuclear cells.** HLA-A2 normal donor peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknica, Durham, NC) as described previously (40). Dendritic cells were prepared using a modification of the procedure described by Sallusto et al. (41).

**Generation of T-cell lines.** Modification of the protocol described by Tsang et al. (42) was used to generate mesothelin-specific CTLs. To generate T-cell line T-1991-P-547, autologous dendritic cells were used as antigen-presenting cells (APC). Autologous nonadherent cells were then added to APCs at an effector-to-APC ratio of 10:1. Cultures were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cultures were then supplemented with recombinant human interleukin 2 at a concentration of 20 units/mL for 7 days; the interleukin 2-containing medium was replenished every 3 days. The 3-day incubation with peptide and 7-day interleukin 2 supplement constituted one *in vitro* stimulation (IVS) cycle. Primary cultures were restimulated with autologous dendritic cells as described above on day 11 to begin the next IVS cycle. Autologous dendritic cells were used as APCs for three IVS cycles. Irradiated (23,000 rad) autologous EBV-transformed B cells were used as APCs after the third IVS cycle. For the restimulation with EBV-transformed B cells, peptides at a concentration of 25  $\mu$ g/mL were used to pulse the autologous EBV-transformed B cells at an effector-to-APC ratio of 1:3 for restimulation. Cultures were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After removal of the peptide-containing medium, the cultures were then supplemented with recombinant human interleukin 2 at a concentration of 20 units/mL for 7 days. T-cell lines from patient 55 (T-55-P-547, T-55-P-554L/556V,

**Table 1.** Binding of human mesothelin peptides and analogue peptides to HLA-A2 molecules

Experiment 1: Analysis of native human mesothelin peptide to HLA-A2 molecules			
Peptide	Amino acid position in mesothelin	Sequence	T2 binding*
P21	21-29	FLLFSLGVW	241 (1.5)
P547	547-556	KLLGPHV <del>E</del> GL	407 (2.6)
MUC-1 (positive control)	NA	ALWGQDVTSV	422 (2.7)
CAP-7 (negative control)	NA	HLFGYSWYK	152

Experiment 2: Analysis of analogue peptides			
Mesothelin agonist peptides derived from P547	Designation	Peptide sequence	T2 binding*
P547 (native)	P547	KLLGPHV <del>E</del> GL	226 (1.5)
554L	P547-1	KLLGPHV <u>L</u> GL	180 (1.2)
554L/556V	P547-2	KLLGPHV <u>L</u> GV	289 (2.0)
548M/554L/556V	P547-3	<u>K</u> MLGPHV <u>L</u> GV	368 (2.6)
548I/554L/556V	P547-4	<u>K</u> /LGPHV <u>L</u> GV	342 (2.3)
548M/554L	P547-5	<u>K</u> MLGPHV <u>L</u> GL	132 (0.9)
548I/554L	P547-6	<u>K</u> /LGPHV <u>L</u> GL	185 (1.3)
547Y/554L/556V	P547-7	<u>Y</u> LLGPHV <u>L</u> GV	234 (1.6)
547Y/554L	P547-8	<u>Y</u> LLGPHV <u>L</u> GL	161 (1.1)
MUC-1 peptide (positive control)		ALWGQDVTSV	390 (2.7)
CAP-7 (negative control)		HLFGYSWYK	144

NOTE: Peptides were used at a concentration of 25 µg/mL. Values in parentheses are fold increases compared with the negative control. Amino acid sequences of the parental P547 peptide (amino acid position 547-556 of mesothelin) and analogue peptides. Amino acids are shown by the single-letter code. Substitution amino acids are indicated in bold italic and underlined.

Abbreviation: NA, not applicable.

\*Results are expressed in mean fluorescence intensity. MUC-1 peptide is an HLA-A2–binding peptide and CAP-7 is an HLA-A3–binding CEA peptide.

T-55-P-548M/554L/556V, T-55-P-548L/554L/556V) were generated by stimulation of PBMCs with autologous dendritic cells pulsed with the P-547 or the agonist peptides using the same stimulation protocol as described above. The markers used for the analysis and identification of dendritic cells were CD11c, MHC-class II, CD80, CD54, CD58, and CD83. CD3 was also used as a negative marker.

**Cytotoxic assay.** Cytotoxicity assays were used as described previously (42).

**Detection of cytokines.** Supernatants of T cells exposed for 24 hours to peptide-pulsed autologous dendritic cells, in interleukin 2–free medium at various peptide concentrations, were screened for secretion of IFN-γ using an ELISA kit (BioSource International, Camarillo, CA) and lymphotactin using an ELISA assay (43). The results were expressed in pg/mL.

**Chemotaxis assay.** Chemotactic responses were examined using the method described previously (44). Briefly, blind Well Chambers (Neuroprobe, Gaithersburg, MD) with polyvinylpyrrolidone-free 5 µm pore size polycarbonate filters previously coated on one side with mouse Collagen IV (Trevigen, Gaithersburg, MD) were used. Supernatants from unstimulated T-55-P547 cells or T-55-P547 cells activated for 24 hours with 25 µg/mL of either P547 or P547-2 peptide were added to the lower chambers, and healthy donor PBMCs ( $7.5 \times 10^4$  cells, 75 µL in complete RPMI medium containing 1% human AB serum) were added to the upper chambers. As negative and positive controls, the lower chambers were loaded with medium alone or recombinant lymphotactin (50 ng/mL in complete RPMI medium containing 1% human AB serum), respectively. After incubation for 4 hours at 37°C, filters were removed from the chambers, fixed, and stained with Diff-Quik stain (Dade Behring, Inc., Newark, DE). Blocking assay was done using anti-lymphotactin antibody (R&D Systems, Inc., Minneapolis, MN). The number of cells associated with the lower side of the membranes was evaluated by direct counting of at

least six ×40 objective fields for the standard samples or at least nine ×40 objective fields for the experimental samples.

**Irradiation of tumor cells.** Human tumor cell lines CFPAC-1, OVCAR-3, and AsPC-1 were irradiated as previously described (45, 46). Tumor cells were harvested in log-growth phase and were placed on ice and irradiated at 10 and 20 Gy by a Cesium-137 source (Gammacell-1000; AECL/Nordion, Kanata, Ontario, Canada) at a dose rate of 0.74 Gy/min. Control samples were also placed on ice but not irradiated. Irradiated and nonirradiated cells were then washed in fresh media and seeded in 75 cm<sup>2</sup> cell culture flasks. Cells were harvested for surface marker determination after 72 hours in culture by flow cytometry.

**Vaccinia virus infection of epidermoid carcinoma cells.** A recombinant vaccinia vector encoding HLA-A2.1 was used for the infection of A431 and A431.H9 cells. This recombinant virus was constructed by the insertion of the HLA-A2.1 gene into the BamH1 J region of the genome of the Wyeth strain of vaccinia virus as described (47). The gene is under control of the vaccinia 40k promoter (48). Target cells at a concentration of  $1 \times 10^7$ /mL in complete RPMI 1640 supplemented with 0.1% bovine serum albumin were incubated with equal volume of vaccinia virus ( $10^8$  plaque-forming units/mL) in the same medium at 37°C for 1 hour. The cells were then adjusted to a concentration of  $5 \times 10^5$ /mL in complete medium and incubated for 3 hours at 37°C.

**Statistical analysis.** Statistical analysis of differences between means was done using a two-tailed paired *t* test (StatView statistical software, Abacus Concepts, Berkeley, CA).

## Results

The primary amino acid sequence of human mesothelin was analyzed for consensus motifs for novel HLA-A2–binding peptides. One 10-mer peptide and one 9-mer peptide were

identified, subsequently synthesized, and investigated for binding to the HLA-A2 molecule in a T2 cell-binding assay. The amino acid sequences and the positions of these peptides are shown in Table 1. The MUC-1 peptide and a carcinoembryonic antigen HLA-A3-binding peptide (CAP-7) were used as positive and negative controls, respectively. Two of the peptides (P21-29 and P547-556) were shown to have positive binding in the T2 assay. Experiments were then conducted to compare the ability of the P21-29 and P547-556 peptides to bind HLA-A2 at various peptide concentrations. As seen in Fig. 1, the P547-556 peptide bound to HLA-A2 at higher levels than did the P21-29 peptide at concentrations of 50, 25, 12.5, and 6.25  $\mu$ g/mL. The P547-556 peptide (designated as P547) was thus chosen for further study.

Studies were then initiated to determine whether mesothelin-specific T-cell lines could be established from PBMCs from an apparently healthy donor. Autologous dendritic cells were used as APCs. The specificity of the mesothelin-specific T cells generated (designated T-1991-P547) was analyzed after IVS cycle 3 (see Materials and Methods) for the ability to release IFN- $\gamma$  and the chemokine lymphotactin after stimulation with autologous dendritic cells pulsed with the P547 peptide. When T-1991-P547 cells were stimulated with autologous dendritic cells pulsed with P547 peptide, the T cells produced 391 pg/mL IFN- $\gamma$  and 78 pg/mL lymphotactin, whereas the use of autologous dendritic cells pulsed with control CAP1-6D peptide or dendritic cells alone did not result in any IFN- $\gamma$  and lymphotactin production (i.e., <16 pg/mL). As determined by flow cytometric analysis, the T-1991-P547 cell line was 98.7% CD8 positive, 62.2% CD45RA positive, 1.1% CD28 positive, and 0.5% CD27 positive. The T-1991-P547 cell line was then analyzed for the ability to lyse mesothelin-positive and HLA-A2-positive human tumor cell lines. AsPC-1 (HLA-

**Table 2.** Ability of T-1991-P547 cells to lyse cancer cells expressing mesothelin

Target	HLA-A2	Mesothelin	Percentage lysis (SD)
CFPAC-1 (pancreatic cancer cells)	100 (222)	93 (21)	34.9 (3.5)*
AsPC-1 (pancreatic cancer cells)	Negative	92 (19)	8.8 (1.3)
OVCAR-3 (ovarian cancer cells)	97 (23)	47.4 (21)	17.6 (2.0)*

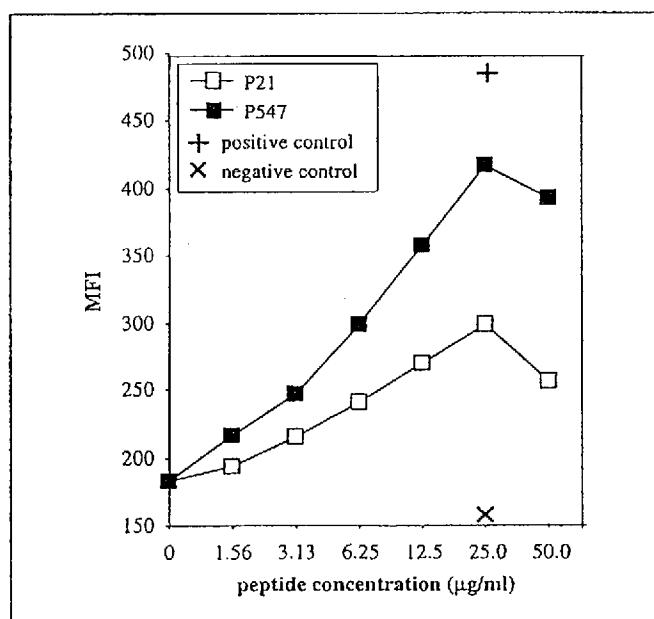
NOTE: HLA-A2 and mesothelin expression were tested by flow cytometry (see Materials and Methods). Results are expressed in percentage of positive cells. A 16-hour  $^{111}\text{In}$  release assay was done. Results are expressed in percentage-specific lysis at effector-to-target ratio of 20:1.

\*Statistical significance ( $P < 0.01$ , two tailed  $t$  test) when comparing lysis of CFPAC-1 cells versus AsPC-1 cells and OVCAR-3 versus AsPC-1 by T-1991-P547 cells.

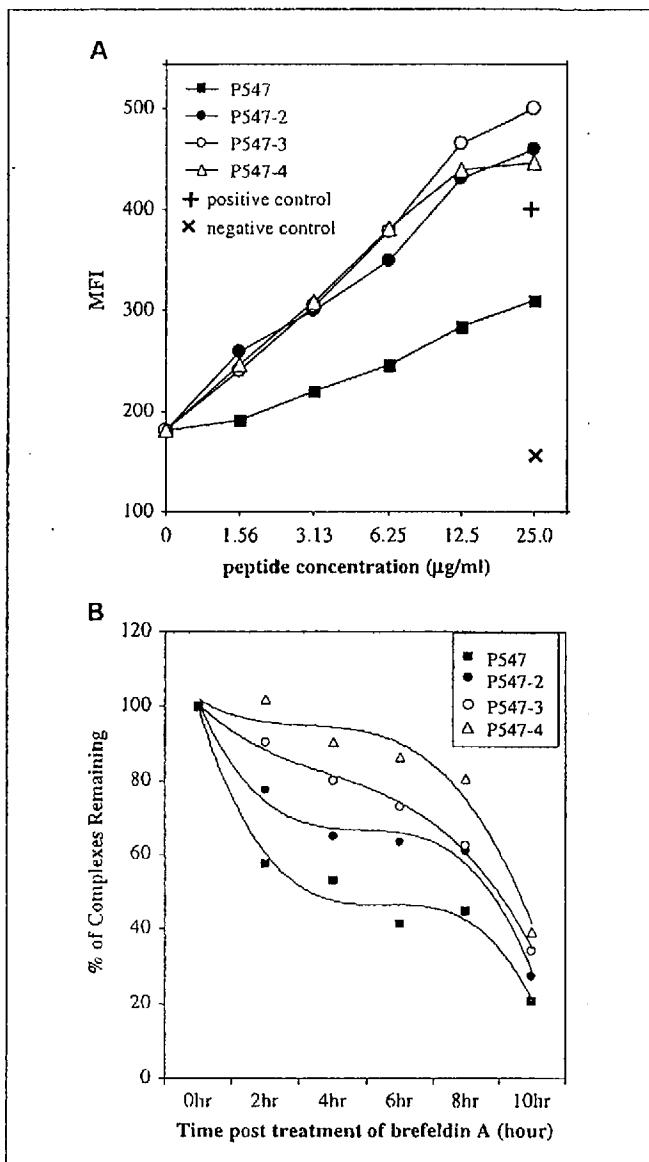
A2-negative and mesothelin-positive pancreatic cancer cell line) was used as a negative control. The expression of HLA-A2 and mesothelin on CFPAC-1, OVCAR-3, AsPC-1, SW1463, and C1R-A2 cell lines was analyzed by flow cytometry. As shown in Table 2, both CFPAC-1 cells and OVCAR-3 cells were lysed by the T-1991-P547 cells. No lysis was observed against AsPC-1 cells. T-1991-P547 cells lysed CFPAC-1 cells to a greater degree compared with OVCAR-3 cells. This may partly be due to the fact that a higher percentage of CFPAC-1 cells were expressing mesothelin compared with OVCAR-3 cells.

Analysis of the primary and secondary HLA-A2-binding anchor amino acid residues at positions 1, 2, 8, and 10 of the P547 peptide revealed that modification of amino acids at these positions could potentially enhance the binding ability of the peptide to the HLA-A2 molecule. For this reason, eight different analogues of P547 peptide were synthesized, as shown in Table 1, and were investigated for their binding ability to T2 cells along with the native P547 peptide. The carcinoembryonic antigen peptide CAP-7, which has previously been shown not to bind to T2 (42), was used as a negative control. As shown in Table 1, three of the eight analogues bound to HLA-A2 at higher levels than the native peptide. They were designated P547-2, P547-3, and P547-4. Experiments were then conducted to compare the ability of these three analogues to bind HLA-A2 at various concentrations. As shown in Fig. 2A, all bound to HLA-A2 at higher levels than did the native P547 peptide at all concentrations. These results indicated that these three analogues with modification in the primary anchor position 2 (position 548 of the mesothelin peptide) and position 10 (position 556 of the mesothelin peptide) as well as the secondary position 8 (position 554 of the mesothelin peptide) were potential agonists of peptide P547.

We then examined the stability of the peptide-MHC complex for P547 (native), and P547-2, P547-3, and P547-4 agonist peptides. The peptides were incubated with T2 cells overnight, and the unbound peptides were washed off and the cells were then incubated with Brefeldin A to block delivery of new class I molecules to the cell surface. Cells were analyzed for the presence of peptide-HLA-A2 complexes at various time points.



**Fig. 1.** Binding of mesothelin peptides to HLA-A2. Peptides were analyzed for binding to T2 cell line as described in Materials and Methods. Peptides were used at concentrations of 0 to 50  $\mu\text{g}/\text{mL}$ . P21 peptide (□), P547 (■), positive control (MUC-1 peptide; +) and negative control (HLA-A3-binding peptide; ×). MFI, mean fluorescence intensity.



**Fig. 2.** Binding of a native mesothelin peptide and its agonist peptides to HLA-A2. Peptides were analyzed for binding to T2 cell line as described in Materials and Methods. *A*, peptides were used at concentrations of 0 to 50  $\mu$ g/mL. P547 peptide (■), P547-2 (●), P547-3 (○), and P547-4 (△), positive control (MUC-1 peptide; +) and negative control (HLA-A3-binding peptide; x). Results are expressed in mean fluorescence intensity. *B*, comparison of the stability of HLA-A2 peptide complexes of mesothelin native and agonist peptides. T2 cells were incubated overnight with P547 (■), P547-2 (●), P547-3 (○), and P547-4 (△) peptides at a concentration of 25  $\mu$ g/mL and then were washed free of unbound peptide and incubated with Brefeldin A to block delivery of new class I molecules to the cell surface. At the indicated times, cells were stained for the presence of surface peptide–HLA-A2 complexes. Points, relative percentage of binding compared with 100% at time 0.

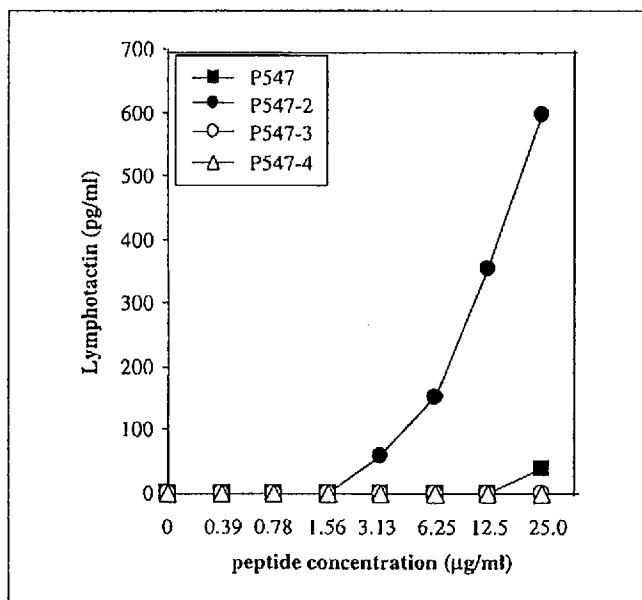
As shown in Fig. 2B, all three HLA-A2 agonist complexes were more stable than the P547-HLA-A2 complex over the 8-hour observation period, with P547-4 slightly more stable than the P547-2 and P547-3 complexes over the same period of time. These data indicated that both the binding to the MHC molecule and the stability of the peptide-MHC complex were greater for the P547-2, P547-3, and P547-4 agonist peptides than the native P547 peptide.

**Table 3.** Production of IFN- $\gamma$  and lymphotactin by T-1991-P547 cells stimulated with autologous dendritic cells pulsed with mesothelin peptide analogues

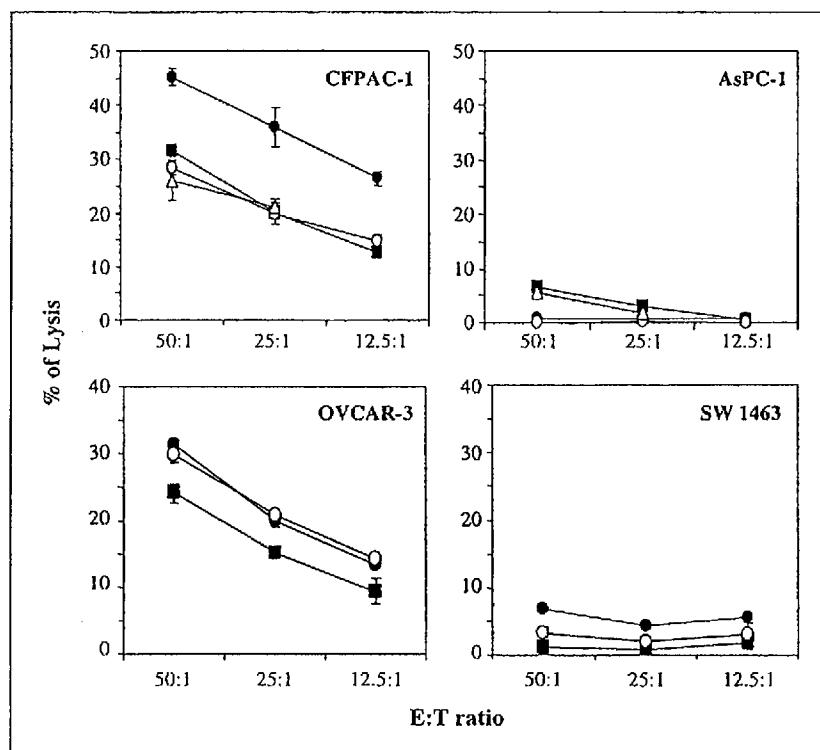
Peptide	Production of	
	IFN- $\gamma$ (pg/mL)	Lymphotactin (pg/mL)
P547	94	49.7
P547-2	449	1,835
P547-3	103	130
P547-4	91	<31.2
None	<15.6	<31.2

NOTE: T-1991-P547 cells were used as effectors in *In vitro* stimulation (IVS-5). T cells were stimulated with irradiated autologous dendritic cells pulsed with different mesothelin analogues at a concentration of 25  $\mu$ g/mL, and an effector-to-APC ratio of 10:1. Twenty-four-hour culture supernatants were collected and screened for the secretion of IFN- $\gamma$  and lymphotactin.

Studies were then undertaken to compare the ability of the P547-2, P547-3, and P547-4 agonist peptides to activate the T-1991-P547 cells, which were generated with the native peptide. As seen in Table 3, pulsing of APCs with P547-2 peptide led to greater levels of both IFN- $\gamma$  and lymphotactin production by T-1991-P547 cells compared with P547-3, P547-4, or the native P547 peptide. Studies were then conducted to compare the ability of the agonist peptides P-547-2, P547-3, P547-4, and the native P547 peptide at various peptide concentrations to activate T-1991-P547 cells in the production of lymphotactin. As shown in Fig. 3, at concentrations of 3.13  $\mu$ g/mL and higher, the pulsing of APCs with P547-2 led to the highest level of lymphotactin production by the T-1991-P547 cell line.



**Fig. 3.** Ability of autologous dendritic cells pulsed with native and agonist mesothelin peptides to induce lymphotactin production by T cells derived from the native peptide (T-1991-P547). Peptides were used at concentrations of 0 to 25  $\mu$ g/mL. Results are expressed in pg/mL.



**Fig. 4.** Ability of the mesothelin-specific T-cell lines established for a pancreatic cancer patient (patient 55) to lyse tumor cells expressing native mesothelin. T-55-P547 (derived from the native peptide; ■), T-55-P547-2 (derived from the agonist peptide P547-2; ●), T-55-P547-3 (derived from the agonist peptide P547-3; ○), and T-55-P547-4 (derived from the agonist peptide P547-4; △). Effector-to-target ratio = 50:1, 25:1, and 12.5:1 in a 16-hour  $^{111}\text{In}$  release assay (see Materials and Methods). Bars, SD.

The T-1991-P547 cell line was derived from an apparently healthy individual using the native P547 peptide. Studies were then conducted to determine whether additional T-cell lines could be generated from a patient with pancreatic cancer (patient 55) using the native or the three agonist peptides. Four mesothelin-specific T-cell lines were subsequently established and were designated T-55-P547, T-55-P547-2, T-55-P547-3, and T-55-P547-4. The T-cell lines were generated by stimulation of PBMCs from patient 55 using autologous dendritic cells. The ability of these T-cell lines to lyse mesothelin-positive and HLA-A2-positive tumor cells was then investigated. As can be seen in Fig. 4, all four T-cell lines were able to lyse the mesothelin-positive and HLA-A2-positive CFPAC-1 cells and OVCAR-3 cells but showed no lysis against AsPC-1 (mesothelin positive, HLA-A2 negative) and SW1463 (mesothelin negative, HLA-A2 positive) cells. These results show the HLA-A2 and mesothelin specificity of the lysis. Furthermore, T-55-P547-2 cells, derived using P547-2 agonist peptide, showed greater lysis of tumor cells than the T cells derived using the native P-547, or the agonist P547-3 and P547-4 peptides. This was seen at three different effector-to-target ratios.

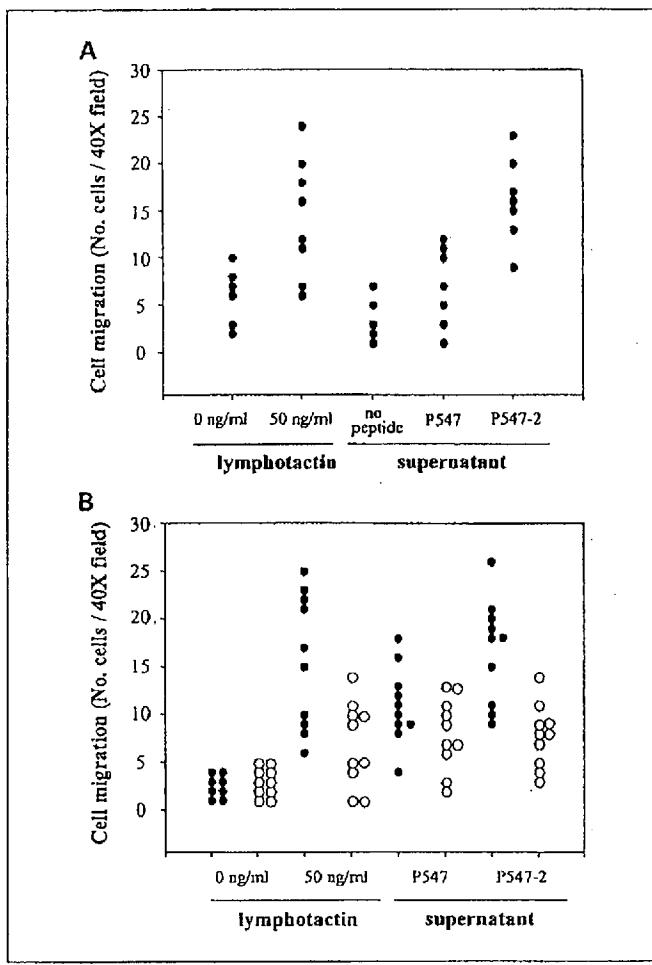
Experiments were then conducted to evaluate the ability of T-55-P547-2, T-55-P547-3, and T-55-P547-4 cells to produce lymphotactin when stimulated with the corresponding peptide. As shown in Table 4, a higher level of lymphotactin production was observed when T-55-P547-2 cells were stimulated with the agonist P547-2 peptide compared with T-55-P547-3, T-55-P547-4, and T-55-P547 when stimulated with the corresponding peptide. Lymphotactin has been reported to enhance chemotaxis responses of various immune cells. A chemotaxis assay was thus done (see Materials and Methods) to confirm the functional activity of the lymphotactin in the supernatants

of the mesothelin-specific T-cell line stimulated with APCs pulsed with the P547-2 agonist peptide. T-55-P547 cells were used in this study. As seen in Fig. 5A, supernatants from T-55-P547 cells stimulated with P547-2 peptide clearly enhanced migration of PBMCs over the responses to the supernatants from the native P547 peptide and the no peptide control. Purified recombinant human lymphotactin was also used as a positive control in the assay. To ascertain that the observed

**Table 4.** Production of lymphotactin by T-cell lines generated from a pancreatic cancer patient (patient 55) stimulated with P547 and the agonist peptides

T-cell line	Production of lymphotactin (pg/mL)	
	Corresponding peptide	CAP1-6D
T-55-P547	<31.2	<31.2
T-55-P547-2	2,843	<31.2
T-55-P547-3	<31.2	<31.2
T-55-P547-4	73.5	<31.2

NOTE: Cells from four mesothelin-specific T-cell lines established from a pancreatic cancer patient (patient 55) were used as effector cells in *in vitro* stimulation (IVS-3). These T-cell lines were established by stimulation with P547-pulsed autologous dendritic cells (T-55-P547), P547-2-pulsed autologous dendritic cells (T-55-P547-2), P547-3-pulsed autologous dendritic cells (T-55-P547-3), or P547-4-pulsed autologous dendritic cells (T-55-P547-4). For lymphotactin production, T-cell lines were stimulated with the corresponding peptide at a concentration of 25  $\mu\text{g}/\text{mL}$  and an effector-to-APC ratio of 10:1. CAP1-6D peptide was used as a negative control. Twenty-four-hour culture supernatants were collected and screened for the secretion of lymphotactin.



**Fig. 5.** Chemotactic responses of human PBMCs to culture supernatant of a mesothelin-specific T-cell line (T-55-P547). *A*, the assay was done using culture supernatant of T-55-P547 cells stimulated with autologous dendritic cells pulsed with either P547 or P547-2 peptide or without peptide for 24 hours. Recombinant lymphotoxin was used at 50 ng/mL as positive control, and medium was used as negative control. Each dot in the scatter plot represents the number of cells in an independently counted field. *B*, the assay was done in the presence (○) or absence (●) of antilymphotoxin antibody at a concentration of 30 µg/mL.

chemotactin responses were due to the presence of lymphotoxin, a chemotaxis assay was done with and without antilymphotoxin antibody. As seen in Fig. 5B, the addition of antilymphotoxin antibody inhibited the migration of PBMCs induced by the purified recombinant lymphotoxin as well as the migration of PBMCs induced by the supernatants from T-55-P547 cells stimulated with APCs pulsed with the agonist peptide P547-2.

To further characterize the P547-2 peptide, an additional T-cell line was established from a colon carcinoma patient (patient 35) using P547-2 agonist peptide-pulsed autologous dendritic cells. This T-cell line was designated T-35-P547-2 and was 97.4% CD8 positive, <1% CD56 positive, 33.1% CD45RA positive, and 29.0% CD27 positive. The T-35-P547-2 cell line was shown to produce IFN- $\gamma$  (100 pg/mL) and lymphotoxin (459 pg/mL) when stimulated with autologous dendritic cells pulsed with P547-2 agonist peptide. As seen in Table 5, the T-35-P547-2 cell line also showed lysis of the mesothelin-positive and HLA-A2-positive CFPAC-1 cells and

OVCAR-3 cells at various effector-to-target ratios but showed no lysis of the mesothelin-positive and HLA-A2-negative AsPC-1 cell line. In addition, the T-35-P547-2 cell line also lysed HLA-A2-positive, mesothelin-positive mesothelioma cell lines YOH, ROB, and ORT, as shown in Table 5. To confirm our hypothesis that tumor cells endogenously process mesothelin to present mesothelin peptide in the context of MHC for T-cell-mediated lysis, CFPAC-1 cells were used as target in a cold target inhibition assay. As shown in Table 6, the addition of peptide-pulsed unlabeled T2 cells decreased the CTL activity of T-35-P547-2 cells against labeled CFPAC-1 cells. The cytotoxic activity of T-35-P547 cells against CFPAC-1 cells was shown to be HLA-A2 restricted as indicated by the inhibition of lysis with anti-HLA-A2 antibody but not with control antibody (Table 6). To further show that target cells can endogenously process the entire mesothelin molecule in a manner so as to bind HLA-A2 molecules for presentation at the cell surface, A431 cells were transfected with the entire human mesothelin gene (see Materials and Methods) and used as target cells in a T-cell cytotoxic assay. As seen in Table 7, the mesothelin-transfected A431 cells designated as A431.H9 cells express mesothelin. A431.H9 cells were susceptible to lysis with T-35-P547-2 cells when transfected with rV-HLA-A2 recombinant expressing HLA-A2. These studies further show the endogenous process of mesothelin in A431.H9 cells and the HLA-A2-restricted nature of the mesothelin-specific lysis of T-35-P547-2 cells.

It has been previously shown that sublethal irradiation of tumor cells can modulate their phenotype to render them more susceptible to T-cell-mediated killing (45, 46). We investigated whether sublethal doses of radiation would alter the expression of surface markers in CFPAC-1, OVCAR-3, and AsPC-1 cell lines. Tumor cells were subjected to 0 or 10 Gy radiation, and cell surface expression of mesothelin, HLA-A2, and intercellular adhesion molecule (ICAM)-1 was analyzed by flow cytometry after 72 hours. No significant increase in cell death was observed with 10 and 20 Gy radiation. As seen in Fig. 6A, slight or no increases in the percentage of cells expressing mesothelin, HLA-A2, and ICAM-1 were detected in the CFPAC-1 cell line at 10 Gy radiation compared with no radiation. This is due to the fact that the CFPAC-1 cell line has a high level of expression of these three molecules. The mean fluorescence intensity of ICAM-1, mesothelin, and HLA-A2, however, did increase (Fig. 6A). As shown in Fig. 6B, increases in the percentage of mesothelin-expressing cells and ICAM-1-expressing cells were observed in the OVCAR-3 cell line after irradiation at 10 Gy. No significant increase in the percentage of cells expressing these three markers was observed on AsPC-1 cells after irradiation at 10 Gy. The mean fluorescence intensity of expression of mesothelin, however, did increase after irradiation (Fig. 6C). The functional significance of phenotypic changes of CFPAC-1 and OVCAR-3 cells after irradiation on the susceptibility to be lysed by mesothelin-specific T cells was investigated. The CFPAC-1 and OVCAR-3 cell lines both showed a significant ( $P < 0.01$ ) increase in lysis by T-1991-P547, a mesothelin-specific T-cell line after 10 Gy of irradiation compared with the nonirradiated CFPAC-1 and OVCAR-3 cells (Fig. 7A and B). The AsPC-1 cell line, a HLA-A2-negative cell line not lysed by T-1991-P547, was used as a negative control (Fig. 7C).

**Table 5.** Ability of the mesothelin-specific T-cell line (T35-P547-2) to lyse human tumor cell lines expressing mesothelin

Target	Type of carcinoma	HLA-A2	Mesothelin	Percentage lysis (SD)		
				50:1	25:1	12.5:1
CFPAC-1	Pancreatic	+	+	29.2 (1.3)*	26.2 (0.4)*	22.8 (0.9)*
OVCAR-3	Ovarian	+	+	25.9 (1.4)*	20.7 (2.2)*	14.9 (0.9)*
AsPC-1	Pancreatic	-	+	2.0 (0.3)	2.7 (0.3)	0.4 (0.4)
YOU	Mesothelioma	+	+	26.0 (1.4)*	26.4 (0.3)*	17.6 (1.2)*
ROB	Mesothelioma	+	+	51.3 (2.4)*	46.1 (0.7)*	31.2 (0.8)*
ORT	Mesothelioma	+	+	49.4 (1.7)*	42.2 (1.7)*	31.0 (0.1)*

NOTE: A 16-hour <sup>111</sup>In release assay was done. Results are expressed in percentage specific lysis at effector-to-target ratios of 50:1, 25:1, and 12.5:1.\*Statistical significance ( $P < 0.01$ , two tailed  $t$  test) when comparing lysis of AsPC cells.

## Discussion

Mesothelin has previously been shown to be a potential target for both antibody- and vaccine-mediated immunotherapy. Preclinical studies using antimesothelin immunotoxin SS1(dsFv)PE38 (SS1P) showed the antitumor activity against mesothelin-expressing tumors (20, 21). Two phase 1 clinical trials using SS1P are currently ongoing (22, 23). Mesothelin-derived HLA-A2-, HLA-A3-, and HLA-A24-restricted CTL

9-mer epitopes have been previously reported (24). These peptides were used for the detection of mesothelin-specific CD8<sup>+</sup> T-cell immune responses in pancreatic cancer patients vaccinated with granulocyte macrophage-colony stimulating factor-transduced pancreatic cancer cells. The two HLA-A2-binding peptides used in that study were mesothelin peptides 20 to 28 (SLLFLLFSL) and 530 to 538 (VPLPLTVAEV; ref. 24). The results from that clinical trial provide evidence that CD8<sup>+</sup> T-cell responses can be generated via cross-presentation by an immunotherapy approach designed to recruit APCs to the vaccination site. In the studies reported here, we have modified the primary anchor residues of a novel mesothelin peptide to augment the binding affinity of the peptide to the MHC molecule. Eight analogues were synthesized and analyzed; one of them, designated P547-2, was shown to be superior to the native epitope in terms of affinity of

**Table 6.** Cytotoxicity of a mesothelin-specific T-cell line (T-35-P547-2) against target cells with endogenous mesothelin expression and inhibition of lysis by anti-HLA-A2 antibody

Target	Percentage lysis (SD)*	Percentage inhibition
Experiment 1: Cold target inhibition <sup>1</sup>		
CFPAC-1	14.3 (0.3)	NA
CFPAC-1 + T2	13.3 (0.6)	NA
CFPAC-1 + T2 + P547-2	1.5 (0.8) <sup>‡</sup>	88.7
Experiment 2: Anti-HLA-A2 antibody inhibition <sup>§</sup>		
CFPAC-1 + anti-HLA-A2 (10 µg/mL)	3.5 (0.1) <sup>  </sup>	72.6
CFPAC-1 + anti-HLA-A2 (50 µg/mL)	3.0 (0.5) <sup>  </sup>	76.4
CFPAC-1 + control antibody (10 µg/mL)	12.7 (0.4)	NA

\*A 16-hour <sup>111</sup>In release assay was done. Results are expressed in percentage specific lysis at an effector-to-target ratio of 20:1.

<sup>1</sup>For the cold target inhibition experiment, labeled CFPAC-1 cells and unlabeled T2 cells were used at a ratio of 1:10. T2 cells were incubated with or without P547-2 peptide (25 µg/mL) in serum-free medium for 24 hours at 37°C before their addition into the assay.

<sup>‡</sup>Statistically significant difference ( $P < 0.01$ , two-tailed  $t$  test) for comparison with CFPAC-1 cells and T2 cells not pulsed with peptide.

<sup>§</sup>For the anti-HLA-A2 antibody inhibition experiment, labeled CFPAC-1 cells were incubated for 1 hour in the presence of medium containing either 10 µg/mL of control antibody (UPC-10) or anti-HLA-A2 antibody (10 µg/mL and 50 µg/mL). Cells were then used as target in 16-hour cytotoxic assays.

<sup>||</sup>Statistically significant difference ( $P < 0.01$ , two-tailed  $t$  test) for comparison with value obtained with control antibody.

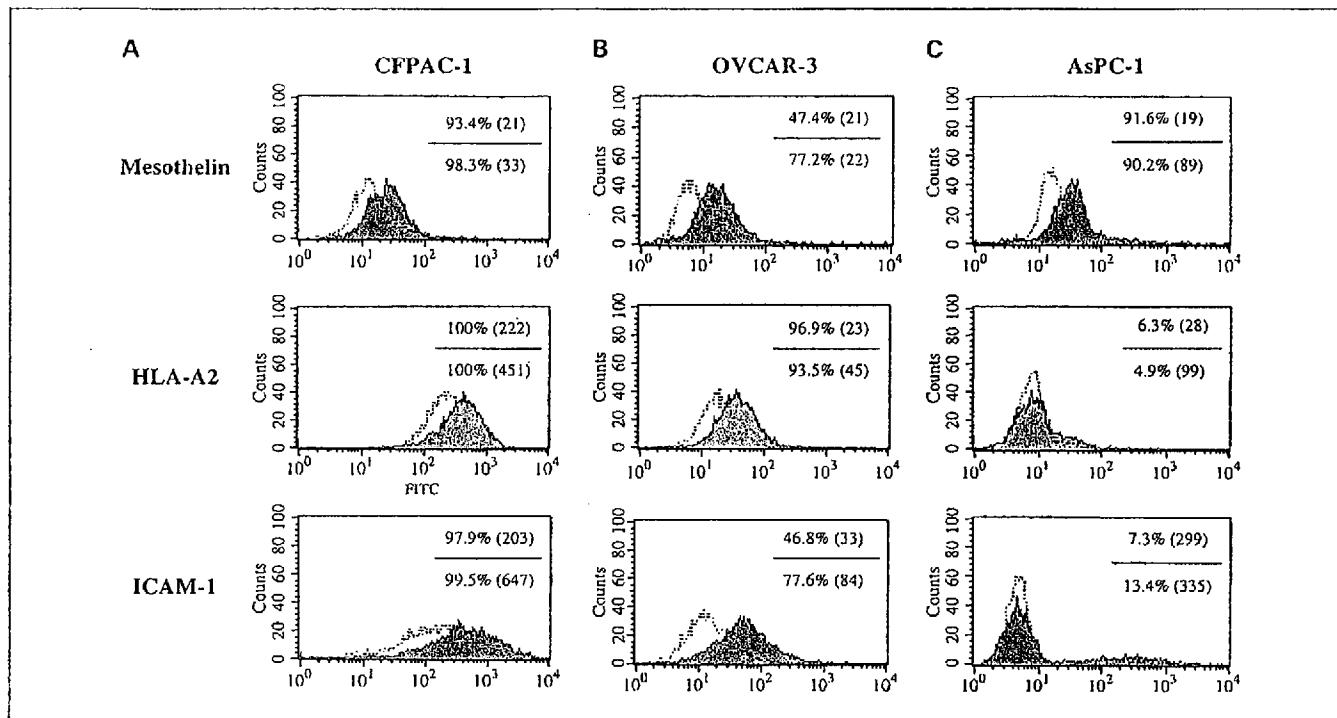
**Table 7.** Demonstration of HLA-A2 involvement in the ability of a mesothelin-specific T-cell line (T-35-P547-2) to lyse target cells with endogenous mesothelin expression

	HLA-A2*	Mesothelin†	% lysis (SD)†
A431			
Uninfected	Negative	Negative	8.5 (1.7)
rV-HLA-A2	95.9 (45)	Negative	9.0 (0.7)
rV-WT	Negative	Negative	6.0 (0.2)
A431.H9			
Uninfected	Negative	99.7 (288)	11.5 (1.4)
rV-HLA-A2	95.9 (55)	80.0 (46)	23.3 (0.01) <sup>‡</sup>
rV-WT	Negative	89.1 (96)	8.0 (0.1)

\*HLA-A2 and mesothelin expression were tested by flow cytometry using anti-HLA-A2 and K1 antibodies, respectively. Values represent the percentage of cells reactive to the antibodies. Numbers in parentheses are the mean fluorescence intensity as determined in relative log units.

†Results are expressed in percentage of specific lysis at an effector-to-target ratio of 40:1.

<sup>‡</sup>Statistically significant lysis compared with rV-WT-infected and uninfected A431.H9 and uninfected A431 as well as rV-HLA-A2-infected and rV-WT-infected A431 cells ( $P < 0.01$ , paired  $t$  test).



**Fig. 6.** Flow cytometry analysis of surface expression of mesothelin, HLA-A2 and ICAM-1 on human tumor cell lines: *A*, CFPAC-1 (pancreatic cancer cells); *B*, OVCAR-3 (ovarian cancer cells); and *C*, AsPC-1 (pancreatic cancer cells) without (unshaded area) and with irradiation at 10 Gy (shaded area).

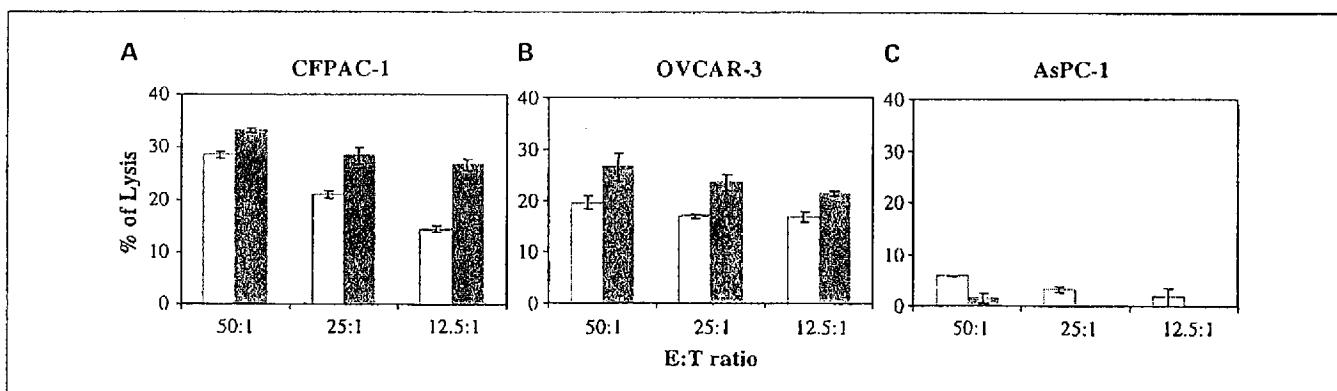
binding to MHC molecules, avidity of the peptide-MHC complex, and the ability to activate CTLs *in vitro*.

T-cell lines derived from the native or the agonist mesothelin epitope were shown to lyse mesothelin-positive and HLA-A2-positive pancreatic cancer, ovarian cancer, and mesothelioma cell lines as well as *mesothelin* gene-transfected epidermoid carcinoma cells in an MHC-restricted manner. Moreover, such T-cell lines could be derived from a pancreatic cancer patient, a colon cancer patient, and a normal donor.

Preclinical and clinical studies have indicated that lymphotactin may be an important chemokine in attracting effector cells and thus enhancing immune responses. The results

reported here show both enhanced synthesis of lymphotactin and enhanced biological activity of lymphotactin as a consequence of stimulation of effector T cells with the agonist mesothelin peptide.

Previous studies have shown that when murine and human tumor cells are exposed to sublethal irradiation, their phenotype can be altered to make them more susceptible to T-cell-mediated killing. This has been shown to be due to up-regulation of either tumor antigen, MHC class I, or accessory molecules such as Fas or ICAM-1, or combinations of the above. The results reported here show that nonlethal irradiation of both a pancreatic and ovarian cancer cell line led to the up-regulation of mesothelin, ICAM-1, and MHC class I, which



**Fig. 7.** Irradiation increases tumor cell sensitivity to mesothelin-specific T-cell killing. *A*, CFPAC-1, a pancreatic cancer cell line. *B*, OVCAR-3, an ovarian cancer cell line. *C*, AsPC-1, a pancreatic cancer cell line. Tumor cells were mock irradiated (white bar) and irradiated with 10 Gy (black bar) and cultured for 72 hours. Tumor cells were labeled with <sup>111</sup>In and were used in a 16-hour <sup>111</sup>In release assay (see Materials and Methods). Bars, SD.

subsequently rendered them more susceptible to lysis by a mesothelin-specific T-cell line.

The studies reported here thus extend previous observations on the suitability of mesothelin as a potential target for immunotherapy of a range of human tumors.

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# Targeted Therapy against Human Lung Cancer in Nude Mice by High-Affinity Recombinant Antimesothelin Single-Chain Fv Immunotoxin<sup>1</sup>

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## Abstract

Several tumors, including mesothelioma and ovarian cancer, can overexpress mesothelin, a glycosylphosphatidylinositol-linked differentiation glycoprotein. The membrane-bound type of mesothelin is found in the blood of cancer patients at a very low level, which makes mesothelin a good candidate for targeted therapy of certain cancers. An antimesothelin disulfide-linked Fv (SS1 Fv) was fused to a truncated mutant of *Pseudomonas* exotoxin A to produce the recombinant immunotoxin SS1(dsFv)-PE38, which has a high binding affinity to mesothelin ( $K_d = 0.7$  nm). Our studies *in vitro* showed that SS1(dsFv)-PE38 is significantly more cytotoxic to the high-mesothelin-producing NCI-H226 human non-small cell lung cancer cells than to human lung adenocarcinoma PC14PE6 cells, which do not express mesothelin. When administered at a nontoxic dose of 500  $\mu$ g/kg on days 7, 9, and 11 to nude mice injected i.v. with the two human lung cancer cell lines, SS1(dsFv)-PE38 selectively inhibited experimental lung metastases produced by the mesothelin-producing NCI-H226 cells. Our data indicate that mesothelin-producing squamous cell carcinoma of the lung may be a good target for this immunotoxin.

## Introduction

Several differentiation antigens such as CD19, CD20, CD22, and CD25 (1–4) that are often preferentially expressed in cancer cells have been targets for therapy in hematopoietic malignancies. Targeted toxin molecules have been con-

structed, and their antitumor activity has been demonstrated (5–8), and several immunotoxins are in clinical trials (5, 9, 10).

One candidate differentiation antigen, mesothelin, is a GPI<sup>3</sup>-linked glycoprotein synthesized as a  $M_r$  69,000 precursor; it is proteolytically processed into a  $M_r$  30,000 NH<sub>2</sub>-terminal secreted form and a  $M_r$  40,000 membrane-bound form (11). The  $M_r$  30,000 secreted form of mesothelin has been termed megakaryocyte-potentiating factor (12, 13). Mesothelin is present only on normal mesothelial cells and on the surface of several tumors, including mesothelioma and ovarian cancer (11, 14, 15). It is not required for normal mouse development or reproduction (16). Its membrane-bound form can be detected in the blood of cancer patients at very low levels, levels too low to interfere with antibody-based therapies (4, 14).

The first antimesothelin monoclonal antibody, K1, was isolated from mice immunized with the ovarian cancer cell line OVCAR3 (15). Although K1 chemically conjugated to a truncated form of *Pseudomonas* exotoxin binds mesothelin-positive cells and cancer cells, it was not useful as an immunotoxin due to poor internalization of the complex (14). Using a phage display library made from spleen mRNA of mice immunized with recombinant mesothelin produced in *Escherichia coli*, an antimesothelin scFv was isolated (17). However, this scFv had a low affinity to mesothelin-positive cells and thus was ineffective for therapeutic targeting. Subsequently, immunization with DNA (18) has been used to immunize mice with a mesothelin expression plasmid (19). A scFv, SS(Fv), was isolated from the splenic RNA of those mice using phage display technology (19). The affinity of this Fv was then improved using a different type of phage display. The resulting Fv was used to construct an immunotoxin by genetically fusing it to a truncated mutant of *Pseudomonas* exotoxin A (20). The purified immunotoxin [SS1(scFv)-PE38] has a high binding affinity to mesothelin, with a dissociation constant ( $K_d$ ) of 0.7 nm. SS1(dsFv)-PE38 is a stabilized form of the Fv in which a disulfide bond connects the light and heavy chain domains of the Fv (20, 21). It is stable at 37°C for up to 40 h. It is cytotoxic to mesothelin-expressing cells *in vitro* and produces antitumor effects against ectopic s.c. cervical epidermoid carcinoma cells that have been stably transfected with the full-length mesothelin cDNA, pcD3CAK1-9 (19).

Our aim was to test this new therapeutic against lung cancer, the leading cause of cancer death for both men and

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<sup>3</sup> The abbreviations used are: GPI, glycosylphosphatidylinositol; scFv, single-chain Fv; NSCLC, non-small cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RFU, relative fluorescence unit(s).

women in the United States. During the year 2001, it was predicted that there would be about 169,500 new cases of lung cancer and that about 157,400 people would die of it (about 90,100 men and 67,300 women). More people die of lung cancer than of colon, breast, and prostate cancers combined (22, 23). We hypothesized that tumor cells that express mesothelin on their surface are susceptible to the SS1(dsFv)-PE38 immunotoxin. In this study we used two different human lung cancer cells that differ in expression of mesothelin. Since the outcome of preclinical cancer therapy depends on the use of orthotopic models (8, 24, 25), we injected the cells i.v. to produce lung lesions. We report that SS1(dsFv)-PE38 immunotoxin inhibited experimental lung metastases produced by mesothelin-expressing human lung cancer cells in nude mice.

#### Materials and Methods

**Cell Line and Culture Conditions.** The NCI-H226 human NSCLC cell line of the squamous type (26) was a gift of Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). The PC14PE6 cell line (27) was isolated from pleural effusions that appeared in a nude mouse injected i.v. with cells from the heterogeneous human lung adenocarcinoma cell line PC14 (obtained from Dr. N. Saito; National Cancer Center Research Institute, Tokyo, Japan). Karyotypic analysis of the PC14PE6 cell line ruled out contamination with murine cells.<sup>4</sup> All tumor cell lines were maintained as adherent monolayer cultures in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), L-glutamine, pyruvate, nonessential amino acids, 2-fold vitamins, and penicillin-streptomycin (Invitrogen, Carlsbad, CA) and incubated in 6.4% CO<sub>2</sub> with balance of air at 37°C. All reagents used for tissue culture were free of endotoxin as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA), and the cell lines were free of *Mycoplasma*, reovirus type 3, pneumonia virus of mice, K virus, encephalitis virus, lymphocyte choriomeningitis virus, ectomelia virus, and lactate dehydrogenase virus (assayed by Science Applications International Corp., Frederick, MD). Cells used in this study were from frozen stocks, and all experiments were carried out within 10 *in vitro* passages after thawing.

**Mice.** Male athymic Ncr-*nu/nu* mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Developmental Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets in facilities approved by The American Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and NIH regulations and standards. The mice (8–10 weeks old) were used in accordance with the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee guidelines.

**Preparation of Immunotoxins.** The immunotoxins SS1(dsFv)-PE38 and anti-Tac(dsFv)-PE38 were constructed by using PCR-based procedures as described previously (20). Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography to near homogeneity as described previously (3, 19, 21). Protein concentrations were determined by the Bradford method (Coomassie Plus; Pierce, Rockford, IL).

**Flow Cytometric Analysis for Expression of Mesothelin.** Log-phase cultures of NCI-H226 and PC14PE6 cell lines were harvested into single-cell suspensions by brief trypsinization. The cells were washed once with medium and once with ice-cold PBS, fixed with 70% methanol at -20°C for 7 min, and then washed with ice-cold PBS. The cells were resuspended in ice-cold 1% BSA in PBS at 1,000,000 cells/ml and then incubated for 45 min at 4°C in the presence of the K1 antibody (15) at 5 µg/ml with intermittent agitation. Cells incubated with the second antibody alone served as negative control. The samples were then washed twice with ice-cold 1% BSA in PBS, reacted with a goat antimouse FITC-labeled F(ab')<sub>2</sub> second antibody (Oncogene Research Products, Boston, MA), and washed with PBS before analysis with an EPICS Profile flow cytometer (Beckman Coulter, Inc., Fullerton, CA; Ref. 28).

**Immunofluorescence Staining for Mesothelin Expression.** NCI-H226 and PC14PE6 cells were plated on sterile Falcon culture slides (BD Bioscience-Discovery Labware, Bedford, MA) in complete Eagle's minimum essential medium containing 10% fetal bovine serum. After 48 h, the slides were washed three times with PBS, fixed in cold acetone for 5 min, and incubated with a protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature. The blocking solution was removed, and the primary K1 antibody (15) was added to the cells at a 1:500 dilution. The immunoreaction was allowed to proceed at 4°C for 18 h. After rinsing four times with PBS and incubating the cells in protein-blocking solution for an additional 10 min, we treated the slides with the appropriate dilution (1:200) of secondary goat antimouse antibody conjugated to Texas Red for 1 h at room temperature in the dark. The samples were washed twice with PBS containing 0.1% Brij and washed with PBS for 5 min. Immunofluorescence microscopy was performed using a ×40 Plan-Neofluar objective (Carl Zeiss, Inc., Thornwood, NY) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel to select for green, red, and blue fluorescence. The expression of mesothelin was identified by red fluorescence, and the images were captured using a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom). Images were further processed using Adobe Photoshop (Adobe Systems, Mountain View, CA) on a computer.

**In Vitro Cytotoxicity Assay.** Because seeding density can influence the results of a cytotoxicity assay, we first determined the optimal density at which to measure growth inhibition of the cell lines, and we found it to be 1200 cells/well for NCI-H226 and 600 cells/well for PC14PE6 cells. After 4 days of growth, this plating density did not produce con-

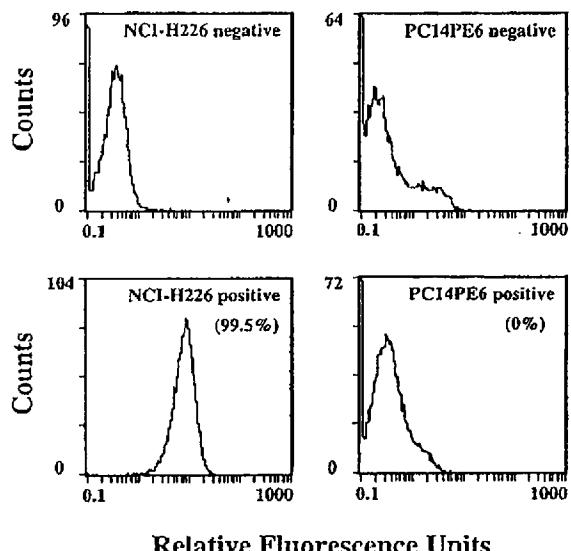
<sup>4</sup> Dr. Sen Pathak, personal communication.

fluence, thus allowing for ready quantitation. In all assays, the tumor cells were seeded in 38-mm<sup>2</sup> wells of flat-bottomed 96-well plates in triplicates and allowed to adhere overnight. The cultures were then washed and refed with medium (negative control) or medium containing various concentrations of the immunotoxins. After 96 h, the antiproliferative activity was determined by the MTT assay (29), which monitors the number of metabolically active cells. After a 2-4-h incubation in medium containing 0.42 mg/ml MTT, the cells were lysed in 100 µl of DMSO. The conversion of MTT to formazan by metabolically active viable cells was monitored by a CERES UV900C 96-well microtiter plate scanner at 570 nm (Bio-Tek Instruments, Inc., Winooski, VT). Survival was calculated from the following formula: survival (%) = (A/B) × 100, where A is the absorbance of treated cells, and B is the absorbance of the control cells.

**Experimental Pulmonary Metastasis.** To prepare tumor cells for inoculation, we harvested log-phase monolayer cultures of NCI-H226 human NSCLC cells by a 2-min exposure to 0.25% trypsin in a 0.1% EDTA/PBS solution. The cells were pipetted gently to produce a single-cell suspension, neutralized with growth medium, washed, and resuspended in calcium- and magnesium-free HBSS. Log-phase monolayer cultures of PC14PE6 human lung adenocarcinoma cells were harvested by repeated pipetting. Cell viability was determined by the trypan blue exclusion method, and only single-cell suspensions of >90% viability were used for the *in vivo* studies. To produce experimental pulmonary metastases,  $2.5 \times 10^5$  NCI-H226 or  $1 \times 10^6$  PC14PE6 cells in 0.2 ml of HBSS were injected into the lateral tail vein of unanesthetized nude mice (30). The i.v. injections of immunotoxins (or control preparations) were administered on days 7, 9, and 11 after tumor cell injection. The mice were euthanized with methoxyflurane when animals in the control group became moribund. The chest wall was resected, and the lungs were removed, weighed, and fixed in Bouin's solution. Tumor lesions were counted under a dissecting microscope.

## Results

**Expression of Mesothelin in Target Cell Lines.** In the first set of experiments, we measured the level of mesothelin expression in several human lung cancer cell lines to select the appropriate target cell line. The levels of mesothelin expression in RFUs are as follows: NCI-H226 squamous cell carcinoma, 134 RFU; NCI-H322 NSCLC, 67 RFU; NCI-H460 large cell lung cancer, 35 RFU; and NCI-H522 NSCLC, 74 RFU. These data indicated that human lung cancers do express mesothelin at a high incidence. We have chosen to use NCI-H226 in this study because of its higher expression of mesothelin. In subsequent experiments, we compared the expression levels of mesothelin between the NCI-H226 human NSCLC cells and the PC14PE6 human lung adenocarcinoma pleural effusion cells. The antimesothelin antibody K1, which was isolated from mice immunized with the ovarian cancer cell line OVCAR3, reacted with 99.5% of NCI-H226 cells and 0% of PC14PE6 cells as determined by flow cytometry (Fig. 1). The difference in mesothelin expression levels of the two cell lines was confirmed by fluorescent



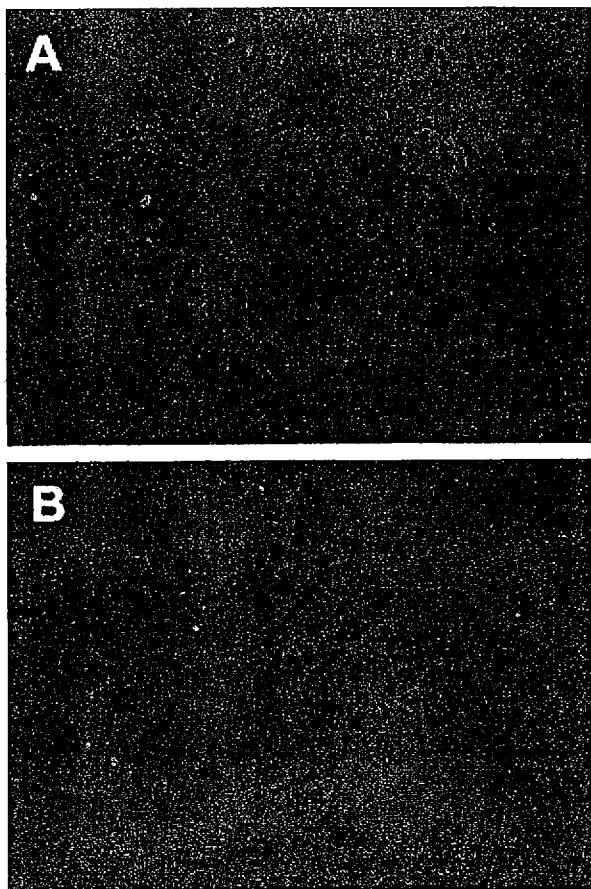
Relative Fluorescence Units

**Fig. 1.** Differential expression of mesothelin by human lung cancer cells. Immunoreactive mesothelin was measured by flow cytometry using the antimesothelin monoclonal antibody K1. Nonspecific immunoreactivity of the cells to a FITC-conjugated secondary goat antimouse antibody was used as negative control. Samples of the NCI-H226 human NSCLC cells consisted of 99.5% mesothelin-positive cells (bottom left panel). The PC14PE6 human adenocarcinoma cells were negative (0%) for mesothelin (bottom right panel). These are representative profiles from one of two measurements.

immunohistochemical staining using the K1 antimesothelin antibody and Texas Red (Fig. 2).

**Differential *In Vitro* Antiproliferative Effect of Immunotoxins.** In the next set of experiments, we tested the effects of SS1(dsFv)-PE38 (antimesothelin immunotoxin) and anti-Tac(dsFv)-PE38 (control immunotoxin) on the NCI-H226 and PC14PE6 human lung cancer cells. Anti-Tac(dsFv)-PE38 was used as a control because it only binds to CD25 and only kills CD25-expressing cells (21). The cells were treated with various concentrations of the immunotoxins for 96 h. Cultures of the mesothelin-expressing NCI-H226 cell line were sensitive to the SS1(dsFv)-PE38 ( $IC_{50} = 0.008 \pm 0.003$  ng/ml, mean  $\pm$  SE;  $n = 3$ ), but not to the anti-Tac(dsFv)-PE38 immunotoxin ( $IC_{50} > 100$  ng/ml; Fig. 3A). The PC14PE6 cells, which do not express mesothelin, did not respond to either immunotoxin ( $IC_{50} \geq 100$  ng/ml; Fig. 3B).

**Therapeutic Effects of Antimesothelin Immunotoxin.** Next, we determined whether SS1(dsFv)-PE38 had a therapeutic effect against experimental lung metastases produced by the NCI-H226 and PC14PE6 human lung cancer cells in nude mice. Groups of mice ( $n = 10$ ) were given i.v. injections of NCI-H226 ( $2.5 \times 10^5$ ) or PC14PE6 ( $1 \times 10^6$ ) cells on day 0. After the tumors were allowed to establish, mice were treated with i.v. administrations of PBS, anti-Tac(dsFv)-PE38 control immunotoxin (10 µg/0.2 ml/mouse), or SS1(dsFv)-PE38 anti-mesothelin immunotoxin (10 µg/0.2 ml/mouse) on days 7, 9, and 11. The tumors of mice injected with the PC14PE6 cells (low-mesothelin-expressing cells) did not respond to either immunotoxin. The tumors of mice injected with the NCI-H226 cells (high-mesothelin-express-

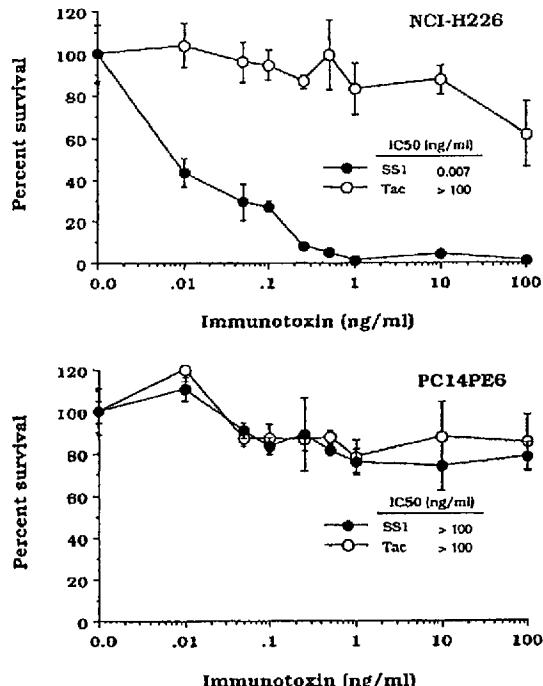


**Fig. 2.** Immunofluorescence staining of mesothelin in human lung target cells. Tumor cells were first reacted with the primary antimesothelin monoclonal antibody K1 at a 1:500 dilution and then incubated with a 1:200 dilution of secondary goat antimouse antibody conjugated to Texas Red. The expression of mesothelin was identified by red fluorescence. The specific immunofluorescence for mesothelin was higher in the NCI-H226 human NSCLC cells (*A*) than in PC14PE6 human adenocarcinoma cells (*B*).

ing cells) responded to SS1(dsFv)-PE38 immunotoxin. The number of lung metastases was significantly reduced from a median of >150 nodules/mouse to a median of 2 nodules/mouse (Table 1). Administration of SS1(dsFv)-PE38 also reduced lung weight (indicative of total tumor volume) in the NCI-H226 tumor-bearing animals; the lung weight of PC14PE6 tumor-bearing animals was not affected by therapy with either immunotoxin (Table 1). These results suggest that the antimesothelin immunotoxin SS1(dsFv)-PE38 produces a specific therapeutic effect against the mesothelin-expressing NCI-H226 cells growing in the lungs of nude mice.

#### Discussion

The membrane-bound form of mesothelin is present on the cell surface of several types of cancer cells (11, 14, 15). In some cancer patients, it is found in the circulation, but at levels that are too low to affect immunotherapy (31). These



**Fig. 3.** *In vitro* dose response of the NCI-H226 human NSCLC cells and PC14PE6 human adenocarcinoma cells to immunotoxins. Cells from exponentially growing cultures were seeded into 96-well tissue culture plates and treated with the indicated concentrations of immunotoxins. Four days later, the antiproliferative effects of the immunotoxins were determined by the MTT assay. The IC<sub>50</sub>s were calculated. Mesothelin-expressing NCI-H226 cells were more sensitive to the cytotoxic effects of the antimesothelin immunotoxin SS1(dsFv)-PE38 than the mesothelin-negative PC14PE6 cells. Neither cell line responded to the anti-Tac(dsFv)-PE38 control immunotoxin (IC<sub>50</sub> > 100 ng/ml). This is one representative experiment of three.

properties of mesothelin expression make it a promising targeting molecule for cancer management. The NCI-H226 human NSCLC cells express a much higher level of mesothelin than the PC14PE6 human lung adenocarcinoma cells. Under *in vitro* conditions, the SS1(dsFv)-PE38 antimesothelin immunotoxin was highly cytotoxic (IC<sub>50</sub> = 0.008 ± 0.003 ng/ml, mean ± SE; *n* = 3) against the NCI-H226 cells, but not against the PC14PE6 cells (IC<sub>50</sub> > 100 ng/ml). The anti-Tac(dsFv)-PE38 (anti-interleukin-2 receptor) control immunotoxin did not produce significant cytotoxicity against either cell line (IC<sub>50</sub> > 100 ng/ml) under *in vitro* conditions. Three consecutive i.v. administrations of the SS1(dsFv)-PE38 immunotoxin eradicated significantly more lung lesions produced by NCI-H226 cells in nude mice than it did those produced by PC14PE6 cells. The specificity of the immunotoxin was confirmed by the lack of effect of the control immunotoxin anti-Tac(dsFv)-PE38 on lung cancer lesions in nude mice.

A critical therapeutic issue for the use of immunotoxins is nonspecific binding to normal tissues, which typically produces toxicity. Mesothelin is a GPI-linked glycoprotein present on the cell surface of normal mesothelial cells. GPI-linked proteins regulate a wide variety of functions in different cells.

**Table 1** Immunotoxin therapy against experimental lung metastases produced by NCI-H226 NSCLC cells or PC14PE6 human adenocarcinoma cells in nude mice

Nude mice were injected i.v. with NCI-H226 ( $2.5 \times 10^6$ ) or PC14PE6 ( $1 \times 10^6$ ) cells on day 0 and treated with i.v. administration of PBS, anti-Tac(dsFv)-PE38 control immunotoxin (10 µg/0.2 ml/mouse), or SS1(dsFv)-PE38 antimesothelin immunotoxin (10 µg/0.2 ml/mouse) on days 7, 9, and 11. Mice injected with NCI-H226 cells were killed on day 51. Mice injected with PC14PE6 cells were killed on day 45. The results shown are representative from one of three independent experiments.

Cell line	Treatment	Lung metastases			Lung weight (mg)	
		Incidence <sup>a</sup>	No.	Median	Range	Median
NCI-H226	PBS control	10/10	>150	68 to >150	252	208–317
	Anti-Tac(dsFv)-PE38	10/10	>150	8 to >150	204	190–264
	SS1(dsFv)-PE38	7/10	2 <sup>b</sup>	0–39	175	141–230
PC14PE6	PBS control	10/10	62	18–91	551	325–1015
	Anti-Tac(dsFv)-PE38	10/10	51	10–83	560	60–920
	SS1(dsFv)-PE38	8/10	65	7–98	540	252–940

<sup>a</sup> Number of mice with tumor/number of mice injected.

<sup>b</sup> P < 0.001 (Mann-Whitney U test).

Many of these proteins are receptors that participate in signal transduction processes; others play a role in cellular recognition and adhesion (16, 32–34). Mesothelin is a differentiation antigen, and it is expressed on the mesothelial cell lining of the lung and in the peritoneal wall of mice, an expression pattern resembling that in human tissues (16). Flat mesothelial cells regulate the movement of molecules and cells in and out of the peritoneal cavity, and mesothelin might have a role in these processes. The  $M_r$  32,000 secretory domain of mesothelin can stimulate megakaryocyte colony-forming activity of murine interleukin 3 in bone marrow cell culture of mice (12, 13, 16). Because SS1(dsFv)-PE38 does not cross-react with mouse mesothelin, the immunotoxin would be more likely to find and bind the human antigen in the animal model. Therefore, a clinical concern exists as to whether normal human mesothelin might act as a shunt for the immunotoxin molecules and hinder the efficacy of SS1(dsFv)-PE38. Mesothelin is present only on normal mesothelial cells and on the surface of several tumors, including mesothelioma and ovarian cancer (11, 14, 15). SS1(dsFv)-PE38 has been constructed against the  $M_r$  40,000 membrane-bound form of mesothelin (11) which can be detected in the blood of cancer patients at very low levels, levels too low to interfere with antibody-based therapies (4, 14). However, therapy with SS1(dsFv)-PE38 might affect normal mesothelin-producing serosal surfaces and cause inflammation or fibrosis, thus limiting the use of the immunotoxin. Future clinical trials could determine the therapeutic index for SS1(dsFv)-PE38 in specific cancer patients.

In summary, we have found that multiple i.v. administrations of recombinant SS1(dsFv)-PE38 antimesothelin immunotoxin produced by fusing the variable regions of a monoclonal antibody directed at the mesothelin in-frame with domain II and III of *Pseudomonas* exotoxin A can significantly decrease the establishment and growth of lung lesions produced by NCI-H226 human NSCLC cells in nude mice. The therapeutic response directly correlated with the expression level of mesothelin on these tumor cells.

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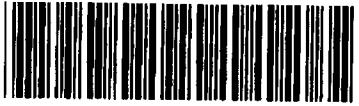
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**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

<b>Application Number</b> 	<b>Application/Control No.</b> 10/495,663	<b>Applicant(s)/Patent under Reexamination</b> PASTAN ET AL. Art Unit Larry R. Helms 1643
<b>Document Code - AP.PRE.DEC</b>		

## Notice of Panel Decision from Pre-Appeal Brief Review



This is in response to the Pre-Appeal Brief Request for Review filed 11/20/07.

1.  **Improper Request** – The Request is improper and a conference will not be held for the following reason(s):

- The Notice of Appeal has not been filed concurrent with the Pre-Appeal Brief Request.
- The request does not include reasons why a review is appropriate.
- A proposed amendment is included with the Pre-Appeal Brief request.
- Other: \_\_\_\_\_

The time period for filing a response continues to run from the receipt date of the Notice of Appeal or from the mail date of the last Office communication, if no Notice of Appeal has been received.

2.  **Proceed to Board of Patent Appeals and Interferences** – A Pre-Appeal Brief conference has been held. The application remains under appeal because there is at least one actual issue for appeal. Applicant is required to submit an appeal brief in accordance with 37 CFR 41.37. The time period for filing an appeal brief will be reset to be one month from mailing this decision, or the balance of the two-month time period running from the receipt of the notice of appeal, whichever is greater. Further, the time period for filing of the appeal brief is extendible under 37 CFR 1.136 based upon the mail date of this decision or the receipt date of the notice of appeal, as applicable.

- The panel has determined the status of the claim(s) is as follows:

Claim(s) allowed: \_\_\_\_\_

Claim(s) objected to: \_\_\_\_\_

Claim(s) rejected: 1,17-21,38,46,48 NOTE : 101 rejection has been dropped 112 first rejection maintained

Claim(s) withdrawn from consideration: \_\_\_\_\_

3.  **Allowable application** – A conference has been held. The rejection is withdrawn and a Notice of Allowance will be mailed. Prosecution on the merits remains closed. No further action is required by applicant at this time.

4.  **Reopen Prosecution** – A conference has been held. The rejection is withdrawn and a new Office action will be mailed. No further action is required by applicant at this time.

All participants:

(1) Larry R. Helms

(3) Bob Wax

(2) Minh-Tam Davis

(4) \_\_\_\_\_

  
**LARRY R. HELMS, PH.D.**  
**SUPERVISORY PATENT EXAMINER**